MITOCHONDRIA IN BIODOSIMETRY: FLOW CYTOMETRY ASSESMENT AND VALIDATION *IN VITRO*

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The JC-1 dye was tested *in vitro* on both established cell lines and peripheral blood lymphocytes after gamma irradiation (IR) to assess its potential in biodosimetric evaluation. In brief, we stained irradiated and non-irradiated cells with the JC-1 dye to determine the existing changes in mitochondrial membrane potential and monitor cell health through flow cytometry. The stage of injury in these cells was evaluated through an irradiated versus non-irradiated ratio (IVNIR), comparing the relative proportion of polarized cells containing red JC-1 aggregates. We observed a proportionally inverse decreasing IVNIR as the radiation dose increased (i.e. 0.5; 1; 2; 4; 6; 8 and 10 Gy), performing the analysis at 4, 8 and 24 h after IR in all the tested cells. The results from the JC1-dye test showed that found that CD4 T lymphocytes were more sensitive to irradiation than other cell types; further, we show the radioresistance of RAMOS cells.

INTRODUCTION

In a nuclear disaster scenario with mass-casualties, the availability of high throughput biodosimetric tools will be necessary to support medical triage. Given the multisystemic nature of radiation injuries, it is unlikely that a single biodosimetric assay can be used as a standalone tool to meet the required demand [(1)]. Thus, searching for new biodosimetric markers and methods, as well as establishing their interoperability, has become a challenging priority in the field of biodosimetry [(2)].

Flow cytometry has already been tested as a high throughput tool and it is routinely used for the multicolor analysis of particles based on biomarker detection [(3)]. One of its advantages lies in the high frequency of analysis, which allows the prompt measurement of thousands of cells in one sample, including several parameters at the same time. Thus, this is a feasible and realistic method for routine blood sample analysis [(4)]. Flow cytometry has been previously used to quantify γH2AX and other markers of ionizing radiation-induced DNA damage, as well in the analysis of lymphocyte immunophenotypes after exposure to IR [(5, 6)]. More recently, imaging flow cytometry has been adapted in the analysis of chromosomal damage in biodosimetry [(7)].

In our study, we use flow cytometry as a tool in the assessment of membrane mitochondrial potential (MMP), which is an important parameter of mitochondrial function and an indicator of cell health [(8)]. Lipophilic fluorescent cation dyes, such as JC-1, are used to measure MMP [(9)]. JC-1 incorporates into the cell's mitochondria, where it forms aggregates. Healthy cells exhibit a pronounced orange-red fluorescence of mitochondrial aggregates detectable by flow cytometry analysis (λ = 590 nm). The collapse of the MMP is shown as decreased orange-red fluorescence and, instead, green fluorescent monomers become detectable (λ = 527 nm).

The aim of our study is to examine mitochondrial membrane potential measured by flow cytometry using the lipophilic cationic probe JC-1 (5,5',6,6'-tetrachloro-l,l',3,3' tetraethylbenzimidazol-carbocyanine iodide) in two cell lines and peripheral blood lymphocytes during 4, 8 and 24 h after exposure to gamma IR. We also evaluated its interoperability with lymphocyte immuno-phenotyping.

MATERIAL AND METHODS

Cell culture

The study included MOLT-4 and RAMOS cells, derived from human T-lymphocyte leukemia cells and Human Burkitt's lymphoma cells, respectively. The cell lines were obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK). The cells were cultured in Iscove modified Dulbecco's medium (IMDM) supplemented with 20% fetal calf serum, 0.05% L-glutamine, 150 UI/ml penicillin, and 50 μg/ml streptomycin (all chemicals from Sigma, St. Louis, MO). The cells were kept in a humidified incubator at 37 °C and controlled 5% CO_2 atmosphere. The cell cultures were split every second day, and sub-cultured at a density of 5×10^6 cells/ml. The cells were used up to a maximum of 20 passages for this study.

Animals

Six clinically healthy Large White piglets of 35 to 42 days of age were purchased from Vemas a.s. (Zamberk, Czech Republic). Blood samples (45 ml) were

collected from the vena jugularis externa into heparinized tubes (20 IU/ml, Zentiva group, Czech Republic).
Animal care and experimental procedures were approved by the Ethical Commission Agreement of the Faculty of Military Health Sciences in Hradec Kralove, Czech Republic and by the Commission for Animal Welfare of the Ministry of Defence of the Czech Republic.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from the blood samples by centrifugation through a Ficoll Histopaque 1077 (Sigma Chemical Co., St. Louis, MO) cushion according to the manufacturer instructions. PBMC were washed once in IMDM, counted and sown at a density of 5×10^6 /1ml IMDM.

Irradiation and sample collection

A total of $5x10^6$ of MOLT-4, RAMOS, or PBMC cells in 1 ml IMDM were used as a reference sample (non-irradiated control samples), whereas that an equal number was dispensed into 24-well polystyrene cultivation plates, in triplicate (Biotech, a.s., Czech Republic). One plate was sham-treated (non-irradiated control), whereas that other plates were individually exposed to a selected dose range (0.5, 1, 2, 4, 6, or 10 Gy) of ^{60}Co gamma radiation (irradiated samples). The control and irradiated plates were then kept in a humidified incubator (37°C, 5% CO2) and harvested at 8, 24 and 48 h after exposure to IR. A positive control ($5x10^5$ /1ml MOLT-4, RAMOS or PBMC cells) was pretreated with 5 µl of Staurosporine (Sigma-Aldrich, Czech Republic) and kept for 4, 8, and 24 hours in a CO_2 humidified atmosphere at 37°C before analysis.
Non-irradiated, non-Staurosporine treated control cells (negative control) ($5x10^5$ /1ml MOLT-4, RAMOS or PBMC cells) were also kept in a humidified incubator (37°C, 5% CO_2) before staining.

JC-1 staining

The JC-1 dye (5,5´,6,6´-tetrachloro-1,1´,3,3´-tetraethylbenzimidazol-carbocyanine iodide); Molecular Probes, Czech Republic) was diluted according to the manufacturer's instructions, after which 5 µM of stock solution were added to 1 ml of 5 $x10^5$ MOLT-4 cells, RAMOS or PBMC cells in IMDM, mixed and incubated in the dark for 20 mins at 21ºC. The cells were washed with 4 ml of PBS and resuspended in 400 µl of PBS.

PBMC fluorescent labeling and flow cytometry

Antibodies

The following mouse anti-pig monoclonal antibodies (moAbs) were used in experiments: anti-CD21 Pacific Blue (CD21 PB; clone LT2; EXBIO, Czech Republic), Swine Workshop Cluster 7/Dyomics 647 (SWC7 Dy647; clone MCA 2313; AbD Serotec, United Kingdom), anti-CD3 Dyomics 647 (CD3 Dy647; clone BB23-8E6; Southern Biotech, USA), anti-CD8α-biotin (CD8α Bi; clone 76-2-11; Southern Biotech) and anti-CD4 PB (CD4 PB; clone 74-12-4, Southern Biotech). The following primary monoclonal antibodies were conjugated with Dy647 (anti-pig CD21 and anti-pig CD3) or with Pacific Blue (anti-pig CD4) by EXBIO custom service (Prague, Czech Republic), the other moAbs were bought conjugated directly from manufacturer.

Labeling protocol

A total of $5x10^6$ PBMCs in 1ml PBS were incubated with a moAbs cocktail: CD21 PB/SWC7 Dy647; CD3 Dy647/ CD4 PB; CD3 Dy647/CD8α Bi (15 min, 4°C), later adding 1µl of Streptavidin conjugated with either APC or Cy7 (CALTAG Laboratories, UK) and incubated in the dark for 15 min at 4°C. The samples were washed twice with PBS, centrifugation and resuspended in 1 ml of IMDM. Afterwards, 5 µl of JC-1 (Molecular Probes, U.S.) were added (final concentration 5 µmol/ l) and incubated in the dark for 20 min at room temperature. The cells were washed once again with 4 ml of PBS, centrifuged and resuspended in 400 µl PBS. 10 µl of propidium iodide (PI, Sigma-Aldrich, Czech Republic) was added to the samples, using a final concentration of 5 µg/ ml, and the samples analyzed by flow cytometry. The data was acquired and analyzed in a CyAn ADP™ flow cytometry analyzer (Beckman Coulter, Czech Republic) using the Summit™ 4.3 acquisition/analysis software.

JC-1 analysis

We used an Irradiated Versus Non-Irradiated Ratio (IVNIR [10]) to compare the relative radiosensitivity of MOLT-4, RAMOS, and PMBC cells, with the latter's subpopulations being analyzed individually. Using an IVNIR, we determined the relative proportion of irradiated cells, or cells cultivated with Staurosporine, against the presence of red JC-1 aggregates in both control and irradiated cells.
IVNIR was estimated across the IR dose range and time points after irradiation utilized in this study. All measurements were performed in at least six repeats and in three different experiments, reflecting the obtained data as mean ± SD. The obtained results were analyzed with a Student's t test.

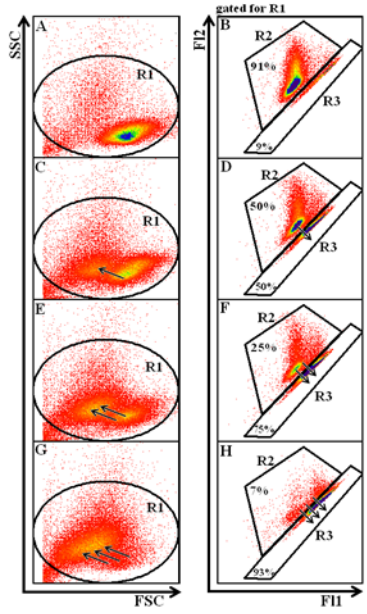
RESULTS

Flow cytometry analysis

We used the JC-1 dye to detect cellular damage induced by gamma IR. The preliminary evaluation of MOLT-4 cells in control, irradiation, and Staurosporine treatment settings can be seen in Figure 1.

MOLT-4 cells (R1 gate, Fig.1 A, C, E and G) are resolved on a size (FSC) and granularity basis (SSC). The cells on the R2 gate were evaluated for the presence of red JC-1 aggregates (Fig. 1 B, D, F, H), which represent live, polarized cells whereas that green JC-1 monomers (R3 gate; Fig. 1 B, D, F, H) represent apoptotic, depolarized cells. We observed a decreasing trend in the relative number of cells with red aggregates (R2): 91% in control (Fig.1 B) to 50% and 25% in irradiated cells (4 and 10 Gy, respectively) (Figures 1D and 1F). Further, there was a significant depletion of red aggregates in the positive control cells incubated with Staurosporine (7%; R2 gate; Fig. 1H). We used this positive control to set a baseline parameter between red aggregates and green monomers during the JC-1 staining analysis.

Figure 1. Representative image of JC-1 staining analysis in MOLT-4 cells irradiated with 4 (C-D) and 10 Gy (E-F) or treated with 5 µM Staurosporine (G-H) and compared to non-



irradiated cells (A-B). All cells were analyzed by flow cytometry after 8 h of cultivation. The relative proportion of live, polarized cells with red JC-1 aggregates of (R2 gate) and depolarized, apoptotic cells with green JC-1 monomers (R3 regions) was estimated from the total number of cells in the R1 gate (A, C, E, G).

While the red aggregates prevailed in the control sample (R2 gate in Fig. 1B), their decreasing trend can be observed as the irradiation dose increases (R2 gate in Figs. 1D and F) or when treated with Staurosporine (R2 gate in Fig. 1H). The cells with lower FSC/SSC characteristics (Figs. 1C, E, and G) share this status. The observed trends are indicated by arrows (Figs. 1C-D, E-F and G-H).

JC-1 staining on MOLT-4 and RAMOS cells

We observed a dose-dependent progression of apoptotic cells during the flow cytometry analysis in the R1 gate in our experiments with MOLT-4 cells. Figs. 2A showing a diminishing number of living cells as the radiation dose progressively increased from 0.5 to 8 Gy, performing the analysis after 24 h of treatment. A similar, although slighter, trend was observed when the analysis was performed after 4 and 8 h of treatment (Fig. 2A).

The RAMOS cells (Fig. 2B), on the other hand, did not display any effect after irradiation in the chosen time points for analysis. The treatment with Staurosporine was effective in both cell lines.

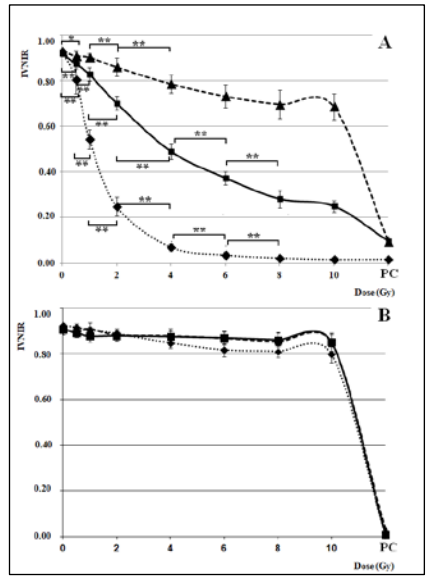


Figure 2. Relative number of living MOLT-4 (A) and RAMOS cells (B) after exposure to ionizing radiation dose (0.5, 1, 2, 4, 6, 8 and 10 Gy). The cells were analyzed after 4h (▲), 8h (■) and 24h (♦) of treatment. Positive control (PC) represents the cells after 4h (♦), 8h (■) and 24h (▲) of treatment with Staurosporine. * $p<0.05$, ** $p<0.01$.

The relative proportion of living cells was estimated for each dose and time point after treatment and it is represented as mean ± SD (n=18), revealing that, in

contrast with RAMOS cells, MOLT-4 cells showed a significant accumulation of green JC-1 monomers after the cells were exposed to the full ionizing radiation dose range used in this study ($p<0.01$).

JC-1 assay on lymphocytes

We performed a similar analysis on the relative proportion (IVNIR) of living PMBC cells based on their FSC and SSC characteristics and accumulation of red JC-1 aggregates. The calculated IVNIR showed a decreasing trend after 24h in all IR dose intervals. However, the cells exposed to cultivation of 4 and 8 h Gy showed a clear IVNIR decline after dose 0.5, 1 and 2 Gy of IR.

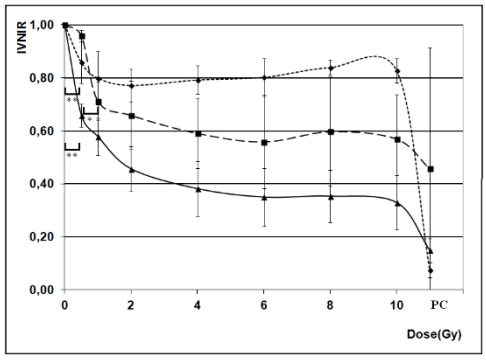


Figure 3. IVNIR (irradiated versus non-irradiated ratio) of lymphocytes analyzed after 4h (♦), 8h (■) and 24 h (▲) of exposure to ionizing radiation. The positive control (PC) represents the cells incubated with Staurosporine.* $p<0.05$, ** $p<0.01$.

The IVNIR value was calculated for every dose and time point and it is represented as mean ± SD (n=18). The difference between the control and the irradiated cells was found highly significant in the lymphocytes analyzed at 4, 8 and 24h ($p<0.01$) and barely significant when comparing the control with the 2, 4, 6, 8 and 10 Gy irradiated cells ($p<0.05$).

The individual radiosensitivity of some lymphocyte subpopulations were also tested, i.e. cytotoxic T cells (Tc; CD3+CD8+) (Fig. 4A-E), T helper cells (Th; CD3+CD4+) (Fig. 4B-F), natural killer cells (NK; CD3-CD8+) (Fig. 4C-G), and B cells (SWC7+CD21+) (Fig. 4D-H) to determine the changes in mitochondrial membrane potential, as reflected by the accumulation of JC-1 dye.

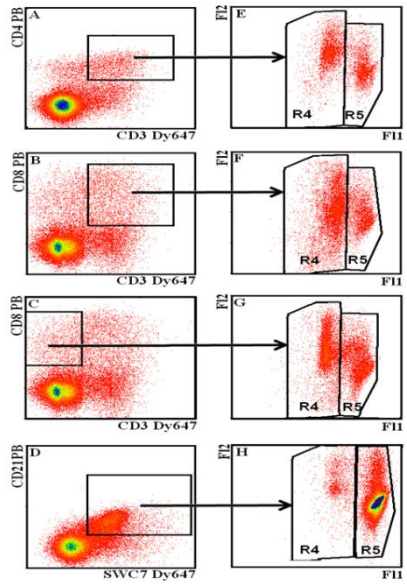


Figure 4. Flow cytometry diagram representing the relative proportion of living, polarized cells with red JC-1 aggregates (R5 gate in E to H) and the dead, depolarized cells with green JC-1monomers (R4 gate in E to H). cytotoxic T cells (**A**); T helper cells (**B**); natural killer cells (**C**), and B cells (**D**).

We evaluated the IVNIR of living, polarized Tc lymphocytes (Fig. 5A), which showed a significantly decreasing trend throughout the IR dose range at both 8 and 24 h after exposure ($p<0.01$). However, this decrement was not even across the board, showing different grades of significance between 0-0.5 Gy ($p<0.05$) and 0.5-2 Gy ($p<0.01$) at 8 h after exposure and 0-1 Gy ($p<0.05$) at 24 h after exposure. The IVNIR values of living, polarized Th cells (Fig. 5B) showed a similar decreasing trend across the used dose range at 8 h after exposure ($p<0.01$) and in the range of 2-10 Gy after 24 h of exposure ($p<0.01$). We found that during low radiation dose triage evaluation, the use of this lymphocyte subpopulation would be suitable in the range of 0-2 Gy at 24 h after irradiation ($p<0.01$).

The IVNIR decline found in NK cells was highly significant throughout the radiation dose range when analyzed at 4 and 8 h after exposure ($p<0.01$). Similar results were obtained at 24 hours after exposure for doses higher than 0.5 Gy ($p<0.05$). Regarding B cells (Fig. 4D), the IVNIR decrement was significant for all irradiation doses and time intervals ($p<0.05$ and $p<0.01$, respectively).

For the purpose of biodosimetric analysis, we suggest the shortest time interval (4 hours) is suitable for all the irradiation doses included in this study ($p<0.01$), as it is in this time interval that we observed the earliest significant differences between the doses of 0.5-1 and 2 Gy ($p<0.01$).

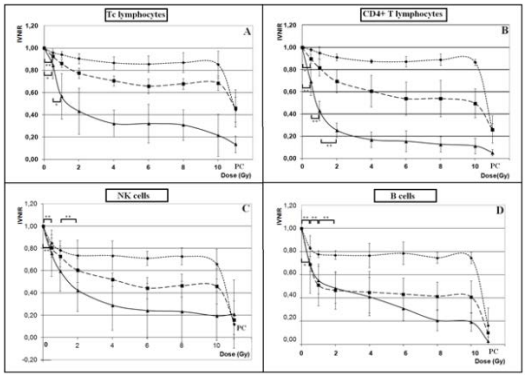


Figure 5. The IVNIR (irradiated versus non-irradiated ratio) measured by the relative proportion of red JC-1 aggregates in cytotoxic T cells (A), T helper cells (B), NK cells (C), and B cells (D), analyzed at 4h (♦), 8h (■) and 24h (▲) after irradiation. The IVNIR value was calculated for every dose and time interval and it is represented as mean ± SD (n=6). All the measured samples showed a statistically significant value when compared with control cells, i.e. $p<0.01$ 8h after irradiation, Tc (4h/1-6Gy/$p<0.01$, 4h/8-10Gy/$p<0.05$, and 24h/4-10Gy/$p<0.01$), Th lymphocytes (4h/2-10Gy/$p<0.01$, and 24h/2-10Gy/$p<0.01$); NK cells (4h/0.5-10Gy/$p<0.01$, 24h/1Gy/$p<0.0$, and 24h/4-10Gy/$p<0.01$), and B cells (4h/0.5Gy/$p<0.05$). 4h/0.5-10Gy/$p<0.01$; 24h/0.5-2Gy/$p<0.05$ and 24h/4-10Gy/$p<0.01$). * $p<0.05$, ** $p<0.01$.

DISCUSSION

In this study we tested both JC-1 and JC-9 dyes, which have similar spectroscopic properties but different chemical composition in the determination of MMP changes after gamma irradiation. Based on our preliminary results we found that, in comparison with the JC-1 dye, JC-9 displays only a weak fluorescent response as the mitochondrial membrane potential declines. For this reason, and for the sake of consistency with other authors (e.g. Salvioli et al. 1997 [11]), we selected lipophilic cationic probe JC-1 in the analysis of the MMP changes in both MOLT-4 and RAMOS cell lines, as well as in different lymphocytic subpopulations in peripheral blood samples.

The MOLT-4 leukemic cell line is highly susceptible to IR; therefore, it was a suitable choice for the analysis of MMP changes after exposure to gamma radiation. The obtained results from the IVNIR analysis showed an increasing radiosensitivity trend, whose dose dependence was tightly correlated with the time elapsed after irradiation, e.g. 1-8 Gy, 0.5-6 Gy or 0.4-4 Gy at 4, 8, and 24h after irradiation, respectively. It must be noted that the sole consideration of Forward versus Side scatter in the viability analysis of MOLT-4 cells [11] could yield biased results, as apoptotic cells can leak into the R1 gate (Figs. 1A, C, E, and G); further, the increment in the relative number of dead cells at higher doses of irradiation is not negligible. In similar manner, the cells classified as “dead” in the R3 gate (Fig. 1B, D, F, H) contained both depolarized according to mitochondrial membrane staining with JC-1. We could confirm the high sensitivity of MOLT-4 cells in the lower range of irradiation doses published by Szekely and Lobreau [12] using the JC-1 staining method.

In some cases, malignant cells are able to withstand high doses of ionizing radiation, such as the Human Burkitt's lymphoma cell line RAMOS [13]. This radioresistance is often caused by the PI3K/AKT signaling transduction pathway, which inhibits IR-induced inhibition of apoptosis [14]. It is for this reason that the RAMOS cell line was used as a negative MMP control in our flow cytometry study (Fig. 2B).

On the other hand, PMBC are widely used in IR studies because of their radiosensitive nature. Therefore, they are often chosen for retrospective estimates in cases of accidental exposure to ionizing radiation. Their relatively fast and low-cost method of isolation is an added bonus [15].

The PBMC cells used in this study MMP included both lymphocytes and monocytes. We found the latter unsuitable for the interpretation of results despite these cells showing measurable and significant MMP changes after 8 and 24 hours of IR exposure, all of which were consistent with our results [16] (data not shown).

Pig lymphocytes are used as a human substitute in radiobiological studies due to the physiological similarity between them [17, 18]. Further, experimental model is characterized by the well-defined phenotypes of lymphocytic subpopulations, including T lymphocytes [19], NK cells [20] and B cells [21].

The effect of gamma radiation on lymphocytes as determined by MMP changes, revealed a significantly decreased IVNIR in the dose range between 0.5 - 1 Gy at 4, 8, and 24 h after exposure. Our results showed a significantly decreased MMP parameter in the analyzed lymphocytes, as calculated by the IVNIR, across the utilized radiation dose range and time intervals when compared with control, non-irradiated cells.

Biodosimetric potential of peripheral blood lymphocyte, particularly of T cell subsets, NK cells and

B cells was tested on peripheral blood in *in vitro* studies. The results from the JC1-dye test showed that Tc lymphocytes were more sensitive to irradiation than other cell types.

Here we show that suitable biodosimeters can also be found in the peripheral blood B-cell compartment. The B lymphocytes have proved useful for retrospective determination of the received dose in the range of 0 - 10 Gy 4 and 24 hours after irradiation.

CONCLUSIONS

We selected the lipophilic cationic probe JC-1 for analysis of MMP changes both in MOLT-4 and RAMOS cell lines as well as in peripheral blood lymphocytes and their subpopulations before and after gamma irradiation. Our study demonstrates that the measurement of MMP potential can represent an appropriate approach for in vitro biodosimetric studies (e.g. testing of radioprotective substances) and other laboratory assays (e.g. viability, cytotoxicity or apoptosis induction).

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REFERENCES

1. Sullivan, J. M., Prasanna, P. G., Grace, M. B., Wathen, L. K., Wallace, R. L., Koerner, J. F. and Coleman, C. N. *Assessment of biodosimetry methods for a mass-casualty radiological incident: medical response and management considerations.* Health Phys. **105**(6), 540-554 (2013).
2. Crespo, R. H., Domene, M. M. and Prieto Rodríguez, M. J. *Biodosimetry and assessment of radiation dose*. Rep. Pract. Oncol. Radiother. **16**(4), 131-137 (2011).
3. Shapiro, H. M., Perlmutter, N. G. and Stein, P. G. *A flow cytometer designed for fluorescence calibration.* Cytometry **33**(2), 280-287 (1998).
4. Mizrahi, O., Shalom, E. I., Baniyash, M. and Klieger, Y. *Quantitative Flow Cytometry: Concerns and Recommendations in Clinic and Research.* Cytometry B Clin. Cytom. **94**(2), 211-218 (2018).
5. Andrejsová, L., Šinkorová, Z., Šinkora, J., Tichý, A., Filipová, A., Němcová, M. and Šinkora, M. *In vivo biodosimetry of porcine T-lymphocyte subsets and NK cells.* Radiat. Prot. Dosimetry **186**(2-3), 181-185 (2019).
6. Zárybnická, L., Vávrová, J., Havelek, R., Tichý, A., Pejchal, J. and Sinkorová, Z. *Lymphocyte subsets and their H2AX phosphorylation in response to in vivo irradiation in rats.* Int. J. Radiat. Biol. **89**(2), 110-117 (2013).
7. Beaton, L. A., Ferrarotto, C., Marro, L., Samiee, S., Malone, S., Grimes, S., Malone, K. and Wilkins, R. C. *Chromosome damage and cell proliferation rates in in vitro irradiated whole blood as markers of late radiation toxicity after radiation therapy to the prostate.* Int. J. Radiat. Oncol. Biol. Phys. **85**(5), 1346-1352 (2013).
8. Krysko, D. V., Roels, F., Leybaert, L. and D'Herde, K. *Mitochondrial transmembrane potential changes support the concept of mitochondrial heterogeneity during apoptosis.* J. Histochem. Cytochem. **49**(10), 1277-1284 (2001).
9. De Biasi, S., Gibellini, L. and Cossarizza, A. *Uncompensated Polychromatic Analysis of Mitochondrial Membrane Potential Using JC-1 and Multilaser Excitation.* Curr. Protoc. Cytom. **72,** 7.32.1-7.32.11 (2015).
10. Řeháková, Z., Sinkora, J., Vlková, M., Vokurková, D., Osterreicher, J., Vávrová, J. and Driák, D. *CD27(+) peripheral blood B-cells are a useful biodosimetric marker in vitro.* Physiol. Res. **57**(4), 589-600 (2008).
11. Szekely, J. G., Lobreau, A. U., Einspenner, M. and Raaphorst, G. P. *The use of flow cytometry to measure X-ray survival in cultured T lymphocytes.* Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med. **47**(6), 681-688 (1985).
12. Szekely, J. G. and Lobreau, A. U. *High radiosensitivity of the MOLT-4 leukaemic cell line.* Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med. **48**(2), 277-84 (1985).
13. Zand, H., Rahimipour, A., Salimi, S. and Shafiee, S. M. *Docosahexaenoic acid sensitizes Ramos cells to Gamma-irradiation-induced apoptosis through involvement of PPAR-gamma activation and NF-kappaB suppression.* Mol. Cell. Biochem. **317**(1-2), +13-120 (2008).
14. Zhan, M. and Han, Z. C. *Phosphatidylinositide 3-kinase/AKT in radiation responses.* Histol. Histopathol. **19**(3), 915-923 (2004).
15. Grievink, H. W., Luisman, T., Kluft, C., Moerland, M. and Malone, K. E. *Comparison of Three Isolation Techniques for human Peripheral Blood Mononuclear Cells: Cell Recovery and Viability, Population Composition, and Cell Functionality* Biopreserv. Biobank **14**(5), 410-415 (2016).
16. Vayssier-Taussat, M., Kreps, S. E., Adrie, C., Dall'Ava, J., Christiani, D. and Polla, B. S. *Mitochondrial membrane potential: a novel biomarker of oxidative environmental stress.* Environ. Health Perspect. **110**(3), 301 -305 (2002).
17. Mittendorf, F., Morczek, A. and Schmidt, W. *Vietnamese miniature pig in radiobiology.* Z. Versuchstierkd **13**(1), 7-16 (1971).
18. Sinkora, J., Reháková, Z., Sinkora, M., Cukrowska, B., Tlaskalová-Hogenová, H., Bianchi, A. T. and De Geus B. *Expression of CD2 on porcine B lymphocytes.* Immunology **95**(3), 443-449 (1998).
19. Saalmüller, A., Kuebart, G., Hollemweguer, E., Chen, Z., Nielsen, J., Zuckermann, F. and Haverson, K. *Summary of*

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workshop findings for porcine T-lymphocyte-specific monoclonal antibodies. Vet. Immunol. Immunopathol. **80**(1-2), 35-52 (2001).
20. Talker, S. C., Käser, T., Reutner, K., Sedlak, C., Mair, K. H., Koinig, H., Graage, R., Viehmann, M., Klingler, E., Ladinig, A., Ritzmann, M., Saalmüller, A. and Gerner, W. *Phenotypic maturation of porcine NK- and T-cell subsets.* Dev. Comp. Immunol. **40**(1), 51-68 (2013).
21. Sinkora, M. and Butler, J. E. *Progress in the use of swine in developmental immunology of B and T lymphocytes.* Dev. Comp. Immunol. **58**,1-17 (2016).
22. Schirrmacher, V. and Zangemeisterwittke, U. *Gamma-irradiation suppresses T-cell mediated protective immunity against a metastatic tumor in the afferent phase of the immune-response but enhances it in the efferent phase when given before immune cell transfer.* Int. J. Oncol. **4**(2), 335-346, 1994.