SCIENTIFIC REPORT

TITLE OF PROJECT: EFFECTS IN UNIRRADIATED CELLS EXPOSED TO PLASMA FROM GAMMA IRRADIATED PERIPHERAL BLOOD

A major part of radiobiology research lately is related to molecular and biochemical changes in cells which did not receive radiation exposure by themselves but were in the vicinity of irradiated cells or were exposed to medium from irradiated cells, i.e. bystander effects. Elucidation of molecular mechanisms underlying bystander effects is essential for evaluation of their contribution to overall radiation damage and may possibly affect risk assessment procedures.

As a research associate at the Bulgarian National Centre of Radiobiology and Radiation Protection I have performed together with my team some preliminary experiments on bystander effects in the case of gamma irradiated whole blood. The results have shown that molecular factors released in the plasma after gamma irradiation of whole blood may lead to DNA damage in bystander leukocyte cells and alter the repair capacity of these cells. This imposed the necessity of including more specific molecular endpoints in the experiments.

That's why I highly appreciated the opportunity to make a short term study visit to the RESC at the DIT and work with Dr. Fiona Lyng. Techniques used in the laboratory of Dr. Lyng allow rapid screening and evaluation of possible molecular signals and processes involved in cellular response to radiation which may result in bystander effects in non-irradiated cells. These and similar techniques may be used to analyze the role of different signal molecules and signal transduction pathways in bystander effects.

The current short term study visit had the following main purposes:

Training in techniques that enable both elucidation of intimate molecular mechanisms as well as rapid evaluation of bystander effects contribution to overall radiation damage. This has provided to me a powerful instrument for my future work both for future scientific research as well as for assessment of the contribution of bystander effects to overall risk assessment for example in the case of occupational exposure to IR which my laboratory at the NCRRP is deeply involved in.

Performing experiments on reporter cell lines that have shown reproducible bystander response after treatment with medium from irradiated cells in order to elucidate if plasma from irradiated whole blood affects leads to bystander effects in these cells; obtain basic information on possible mechanisms, i.e. biochemical pathways involved in bystander effects in the case of irradiated blood which is essential for shaping ideas for future experiments.

The aim of the current project is to evaluate and characterize bystander effects after exposing cells to plasma from gamma irradiated peripheral blood. For that reason the following tasks were put:

1. To determine the cytotoxicity of bystander factors released in the blood plasma after irradiation of peripheral blood samples by clonogenic assay.

2. To determine how plasma from irradiated blood affects cellular metabolic activity by evaluation of cell viability.

3. To evaluate the role of possible molecular signals and processes involved in cellular response to radiation which may result in bystander effects in non-irradiated cells by blocking certain biochemical pathways and evaluation of cell metabolic activity.

4. To determine the role of mitochondria in bystander effects in the case of blood plasma transfer by evaluation of Mitochondrial Membrane Potential after treatment.

5. To determine calcium signaling events in non-irradiated cells exposed to plasma from irradiated blood by imaging of calcium fluxes.

Description of the work:

Cell line and cell culture:

For all experiments the HPV-G cell line was used - a reporter cell line which has shown a reproducible bystander response of approximately 40% reduction in cloning efficiency over a wide range of radiation doses and well characterized calcium fluxes and mitochondrial effects. HPV-G cells were cultured in Dulbecco's MEM : F12 (1:1) medium containing, 10% fetal calf serum 1% penicillin-streptomycin solution 1000 IU, 2mM L- glutamine and 1µg/ml hydrocortisone. Cells were maintained in an incubator at 37ºC, with 95% humidity and 5% $C0_2$.

Blood plasma:

Peripheral human blood from three healthy donors was taken (approx. 32 ml per donor). 16 ml of blood from each donor were gamma irradiated with 0.5 Gy using a Cobalt-60 unit at a dose rate of approximately 1.4 Gy/min. 16 ml from each donor were left as a control. Control tubes were sham irradiated. The source to sample distance was 80 cm. Plasma was isolated two hours after irradiation by centrigugation at 1700 rcf for 12 min.

Clonogenic assays:

Since plasma can be toxic to some cell lines preliminary experiments were carried out in order to test toxicity to HPV-G cells. HPV-G cells were plated in 35 mm petri dishes at cloning densities (approx 400 cells) and exposed to plasma for 24 hours. Cells were then incubated in medium and allowed to form colonies for 7 days. Very few colonies (4 – 10) were formed in all treated with plasma petri dishes with no difference between irradiated and non-irradiated plasma compared to the untreated medium control dishes (58-78 colonies).

For further experiments plasma was diluted to medium 1:4. Cells were treated and incubated to form colonies for 7 days without changing the medium. Incubation was carried out in a humidified 37°C incubator in an atmosphere of 5% CO_2 .

Alamar Blue Assay

Microplate assays with specific fluorescent dyes were performed, the so called Alamar Blue Assay. This is a quick, efficient and reliable method to measure proliferation and cytotoxicity of cells by measuring their innate metabolic activity. The oxidised, non-fluorescing form is reduced by cellular dehydrogenases to fluorescing pink form which is due to intracellular enzyme activity of oxygen consumption during metabolism. Fluorescence can be then measured spectrophotometrically.

4000 cells were plated per well of a 96 well plate and treated with plasma diluted with medium (plasma: medium = 1:4) for 96 hours. Cell viability was evaluated .

Alamar Blue Assay in the presence of ROS, MAPK and JNK pathway inhibitors was performed to determine the role of these biochemical pathways in bystander processes. Inhibitors were:

ROS inhibitor – SOD (superoxide dismutase) at a final concentration of 100 µg/ml;
MAPK (mitogen-activated protein kinase) inhibitor - PD98059, inhibitor of the ERK signalling pathway at a final concentration of 20µM;
SP600125 – inhibitor of JNK pathway at a final concentration of 10µM.

Calcium imaging

Calcium flux analysis was performed using confocal laser scanning microscopy Intracellular calcium levels were determined relative to control levels using two visible wavelength calcium sensitive dyes, Fluo 3 and Fura Red. Fluo 3 exhibits an increase in green fluorescence upon binding to calcium whereas Fura Red exhibits a decrease in red fluorescence upon binding to calcium. The ratio Fluo 3 / Fura Red is a good indicator of intracellular calcium levels. Cells were loaded with the calcium sensitive dyes by incubation with 3 µM Fluo 3 and 3 µM Fura Red AM esters for 1 hour in the buffer at 37^{o}C. Subsequently, the cultures were washed three times with buffer. Fluo 3 and Fura Red were excited at 488 nm and fluorescence emissions at 525 nm and 660 nm were recorded simultaneously using a Zeiss LSM 510 confocal microscope. Ratio images and time series data of the Fluo 3 / Fura Red fluorescence intensities were recorded every 2 seconds. Blood plasma was added after 10 seconds when a stable baseline had been established.

Mitochondrial membrane potential analysis

Mitochondrial membrane potential was measured using a green fluorescent dye, Rhodamine 123 which accumulates in active mitochondria with high mitochondrial membrane potential. HPV-G cells were seeded at high concentrations (approx. 3 x 10^5 cells) on glass coverslips, and incubated in normal culture medium until the cells had attached. This medium was then removed and cells were exposed to plasma from irradiated or non-irradiated blood from each donor 6 hours at 37ºC. The plasma was then removed and cells were washed with buffer and loaded with 5µM Rhodamine 123 for 15 minutes at 37ºC. The dye was then removed, the cells were washed in Ca^{2+} / Mg^{2+} buffer twice and the coverslips were inserted into custom designed petri dishes, which allowed imaging through the glass coverslip in the base of the dish. Using a Zeiss LSM confocal microscope the HPV-G cells were excited at 488nm and fluorescence emission was recorded at 525nm.

Statistical analysis

Statistical analysis of the obtained results was carried out using standard statistical operations and software tools.

Main Results:

1. Clonogenic assay.

Table 1*. Number of colonies after 7 day incubation with diluted plasma for irradiated and non-irradiated blood plasma and for each donor*

	Plasma from non-irradiated blood				Plasma from 0.5 Gy irradiated blood			
	Sample1	Sample2	Sample3	Average	Sample1	Sample2	Sample3	Average
Donor1	103	102	-	102±1	77	96	-	86±13
Donor2	104	88	101	98±8	90	95	65	83±16
Donor3	85	90	103	93±9	55	92	74	74±18

Medium control samples that haven't undergone any treatment with plasma, neither irradiated nor non-irradiated, developed an average of 95 colonies for 7 days. For that reason we accepted that diluted plasma (plasma: medium = 1: 4) shows no toxicity to HPV-G cells.

The clonogenic assay shows a reduction in clonogenic survival of cells treated with plasma from irradiated blood compared to controls treated with plasma from non-irradiated blood, although not statistically significant. The number of samples was limited due to the large quantity of blood plasma needed for each sample.

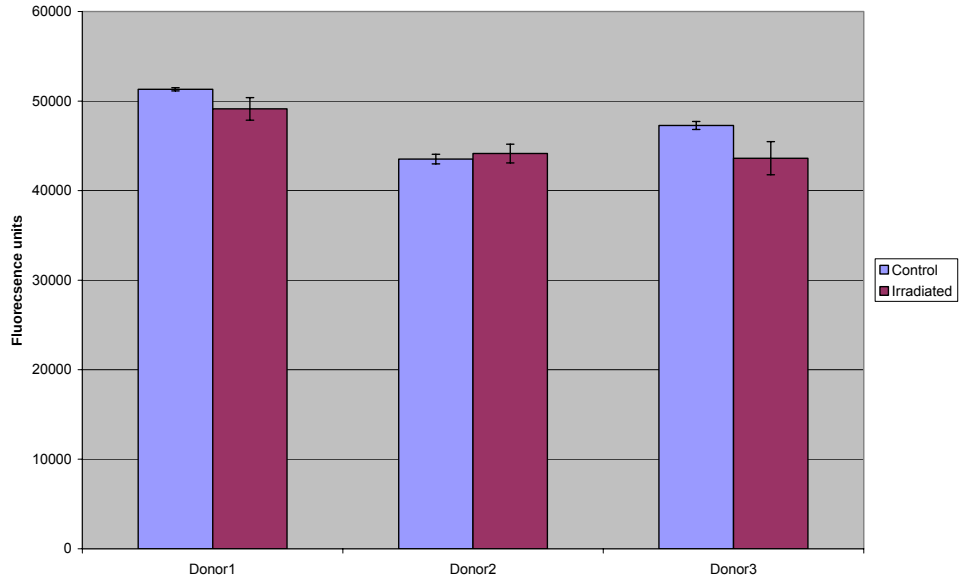
2. Alamar Blue Assay

Table 2. *Viability presented relative to the unirradiated control for each donor*

	Non-irr control (C)	0.5Gy (irr)	C+SOD	irr+SOD	C+MAPK inh	irr+MAPK inh	C+JNK inh	irr+JNK inh
donor1	1	0.96	0.98	0.98	0.88	0.83	0.85	0.67
donor2	1	1.01	1.03	1.05	0.88	0.97	0.95	0.86
donor3	1	0.92	0.96	0.90	0.90	0.83	0.94	0.90

It was observed that treatment with blood plasma from the three donors leads to different effect on the cells with the only statistically significant effect seen for donor 3 (figure 1). Little effect is observed when cells are treated with plasma from donor 1 and practically no effect for donor 2.

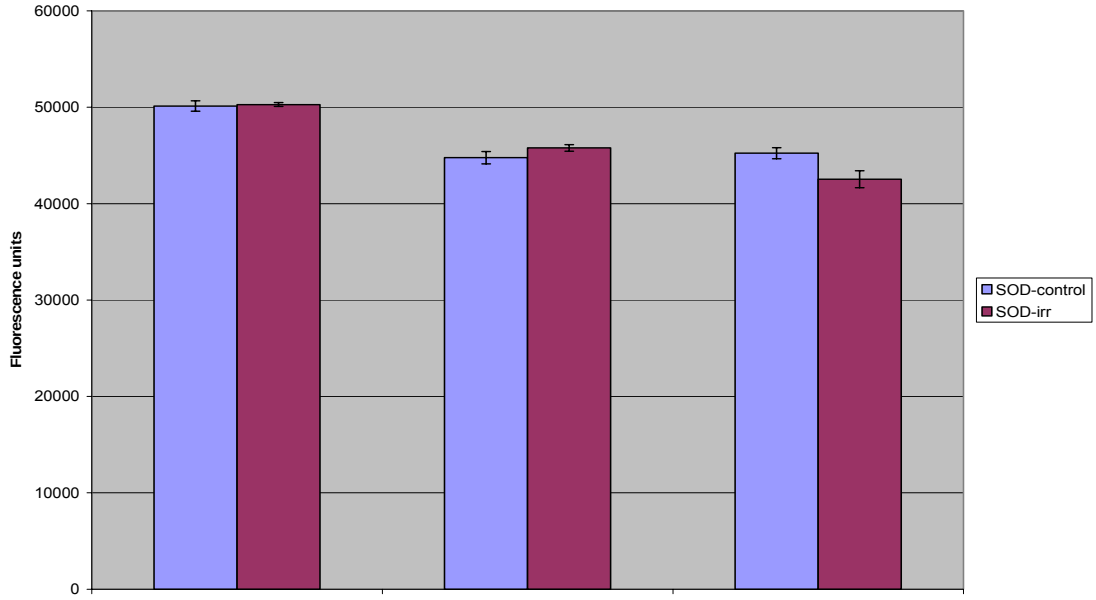
Figure 1. *Cell viability after treatment with plasma from non-irradiated and irradiated blood*



After adding inhibitors of different biochemical pathways, measurements of metabolic activity has shown that cells again react differently to blood plasma from different donors.

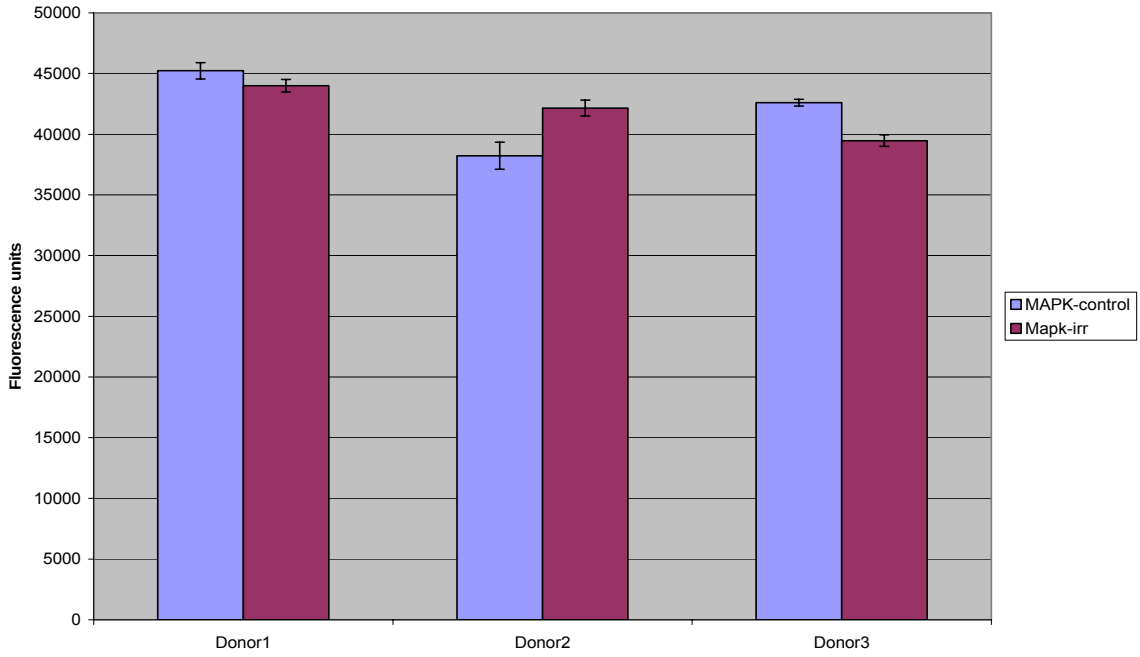
In the presence of an inhibitor of Reactive Oxygen Species (ROS), no difference in viability was observed between non-irradiated and irradiated plasma for the donor 1 and donor 2, although before adding superoxide dismutase (SOD) some bystander effects were observed for donor 1 (fig.2). It may be suggested that bystander effects have been blocked by SOD in the case of that donor. In the case of the donor 3 however bystander effects were not blocked by SOD which may suggest that ROS formation is not an essential pathway for exerting bystander effects in this case.

Figure 2. *Cell viability in the presence of SOD*



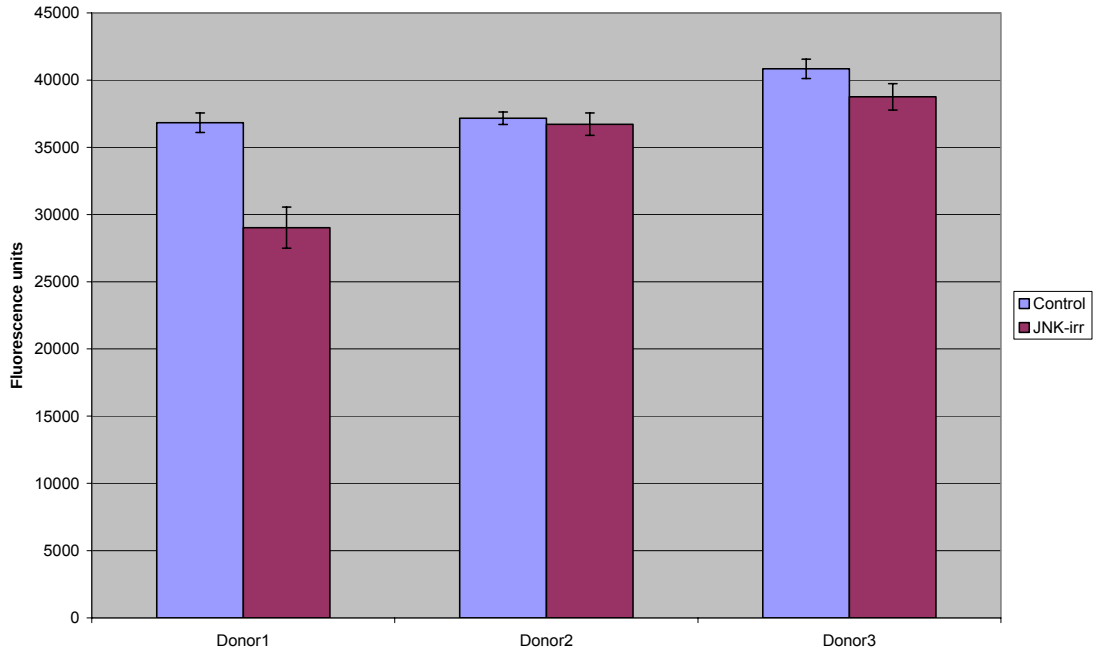
Inhibitors of MAPK biochemical pathways were shown to very slightly reduce the observed bystander effect in the case of the donor 1, but not in the case of the donor 3 (fig.3). This correlates well with the results from the SOD experiment.

Figure 3. *Cell viability in the presence of MAPK pathways inhibitor*



Incubating the cells with blood plasma in the presence of an inhibitor of JNK biochemical pathways have shown that like the other pathways investigated this one also appeared not to be involved in inducing bystander effects in the case of the donor 3. Effects could still be observed in spite of the fact that JNK pathways were blocked. Interestingly, in the case of the donor 1 blocking the JNK pathway has led an even larger bystander effect (fig. 4).

Figure 4. *Cell viability in the presence of JNK pathways inhibitor*



This study has highlighted the individual variation in bystander factor production between the three donors. In addition modulation of the effect by inhibitors appears to vary in each donor.

3. Calcium fluxes imaging

Confocal microscopy have shown that no calcium fluxes were induced by adding plasma from non-irradiated blood. Overall effect in the case of plasma from irradiated blood from all three donors was not observed as well. However, after analysis of changes in certain regions of the field it was observed that certain cells do respond with calcium signaling to addition of irradiated plasma. Evidence of calcium signaling in individual cells after addition of non-irradiated plasma was not found. Differences between the three donors were not observed as well (fig. 5 and 6).

Figure 5. *Fluo 3/Fura Red ratio alteration with time. The overall effect is shown as well as the effect for 3 different cells.*

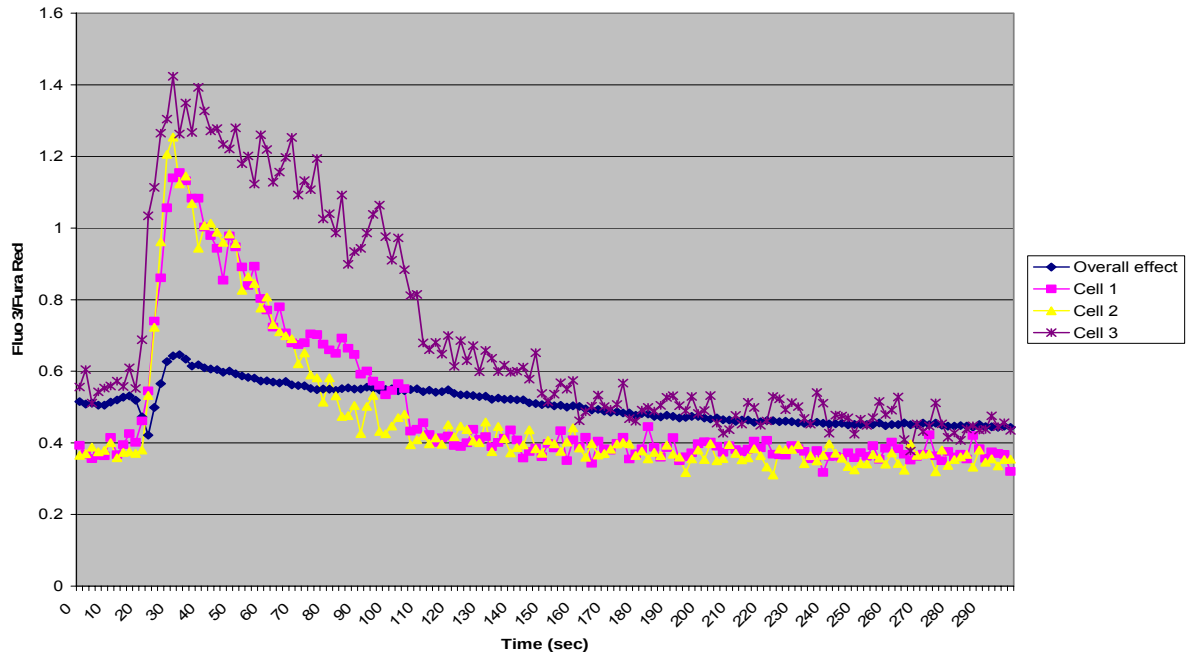
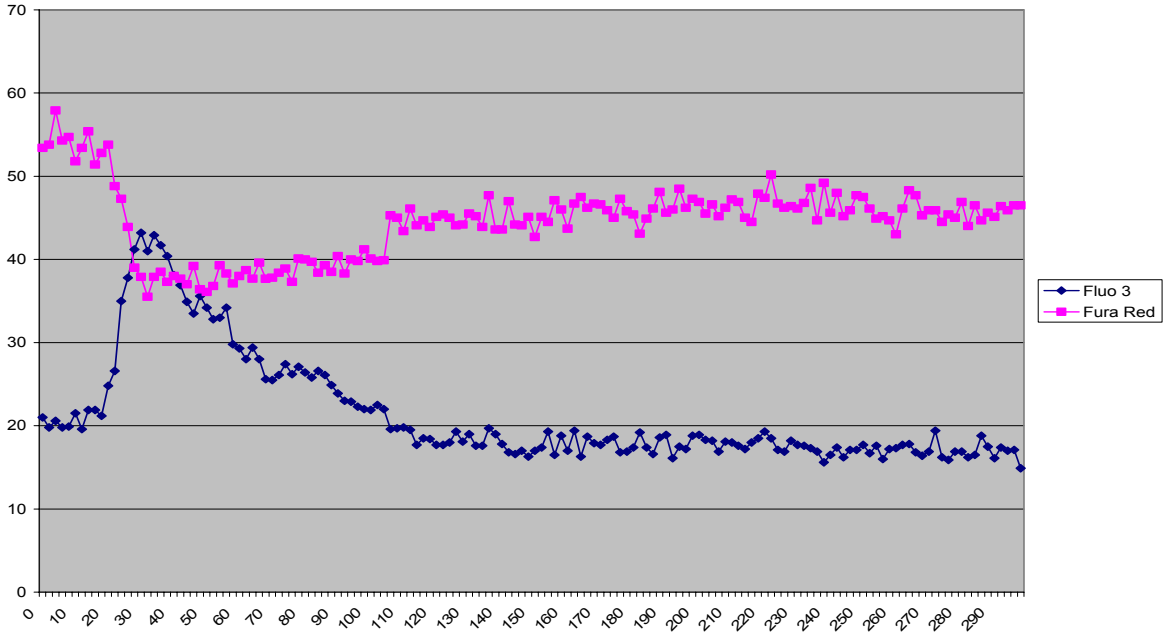


Figure 6. *Fluorescence intensity of the calcium sensitive dyes Fluo 3 and Fura Red in an individual cell in response to addition of plasma from irradiated blood.*

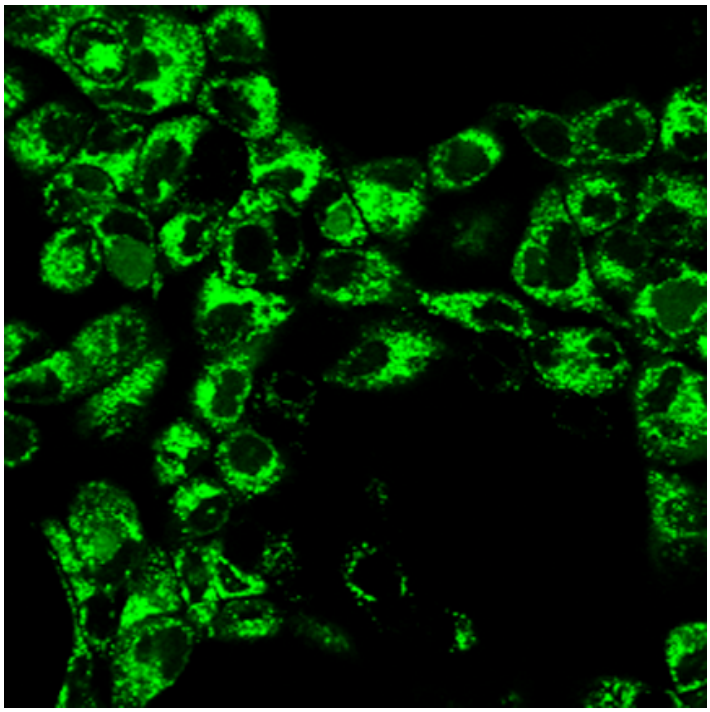


The data shown are representative of all similar plots.

4. Mitochondrial Membrane Potential.

Mitochondrial Membrane Potential analysis was the last experiment we have performed, in order to investigate the involvement of mitochondria in bystander effects induced by blood plasma. Unfortunately, we consider the experiment to be unsuccessful and are not able to make any conclusions based on the results. Obviously, the cells have lost their viability and were not in their normal metabolically active condition at the time they underwent treatment, the reasons for which we have not been able to elucidate. Instead of accumulating in active mitochondria which should give a clear image of concentrated fluorescence centers, Rhodamine 123 seemed to be uniformly distributed in the cells (fig.7). Due to the limited amount of plasma that could be obtained from one donor, the experiment could not be repeated. However, Mitochondrial Membrane Potential analysis via Rhodamine 123 fluorescence measurement will take place in our further research on plasma induced bystander effects as well as in different fields of my future research.

Fig.7. *Non-typical, uniform distribution of Rhodamine 123, image from confocal microscopy*



Conclusions:

1. The current investigation indicated that radiation induced bystander effects after irradiation of whole blood strongly vary between individuals. Therefore, a different contribution of bystander effects to overall radiation induced effects may be suggested depending on individual variations.
2. Individual variations may be due to differences in the activity of biochemical pathways involved in bystander effects. For that reason, a variation of possibilities for modulation of bystander effects may exist in the case of different individuals.
3. Cellular response to treatment with plasma from irradiated blood may be found in individual cells even if overall effect is not observed. Therefore, in addition to different pathways involved, a different number of cells responding with signaling events may be the reason for individual variations.

We consider the results shown here as preliminary and intend to use this project as a basis for more serious and detailed research. The fact that plasma from 0.5 Gy gamma irradiated whole blood can exert bystander effects in a reporter bystander cell line may lead to the assumption that bystander effects may contribute to radiation risk after *in vivo* irradiation with low doses, for example in the case of occupationally exposed individuals. Future collaboration between the RESC at Focas Institute and the Bulgarian National Center of Radiobiology and Radiation Protection is planned. This will include evaluation of bystander effects in radiosensitive cells of individuals exposed to ionizing radiation at their workplace and their contribution to overall radiation risk; investigation of possible biochemical mechanisms of bystander effects in the case of *in vivo* irradiation with low doses at low dose rates; investigation of the possibilities for modulation of bystander effects after *in vivo* irradiation with low doses at low dose rates.