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# Cosmic Radiation on Human Estrogen-Dependent Epithelial Cells. The Lessons from Transmediterranean Flights on Stratospheric Balloons

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## Introduction

In vitro cellular systems are particularly important models for studying radiation effects and understanding the molecular damages caused by such physical mutagens. Ionizing radiation is of fundamental interest for space missions and particularly in view of the «International Space Station» utilization. The fate of cells exposed to outer space radiation may definitely depend from the modification of gene expression levels. Only an exhaustive perception of the induction/repression profiles of specific genes following exposure of cells to radiation will help to disclose the mechanisms that govern the cellular response to injury.

We know that microgravity and radiation alter the mytogenic activation of T lymphocytes and alter the gene expression and function of other cells [1]. However it is not known yet if such alteration depends upon the sole action of microgravity, the sole action of cosmic radiation or both. To answer to these questions we took advantage from two transmditerranan flights (named BIRBA I, 2000 and BIRBA II, 2002, respectively) organized and financed by the Agenzia Spaziale Italiana. During such missions human estrogen-dependent epithelial cells lines, embarked on Stratospheric Balloons, touched 40,000 meters of altitude while flying from Sicily (Italy) to Sevilla (Spain) and were hit by cosmic radiation. The use of this cellular system may even ultimately support or deny the circulating (but unproven) hypothesis that the cosmic radiation negatively affects the delicate equilibrium of the endometrial (and mammary) tissue of female crew members recruited for long space missions. To this purpose we evaluated (by western blots) changes in the expression levels of various proteins which included Bcl-XL, Bcl-2 and Bax (apoptosis), p21, pRb, p53, cyclines A and B and c-kinases Cdk2 and Cdc2 (cell cycle) and HSP 70 (stress). These evaluations have been compared to those obtained from the same cells hit with artificial radiation, namely both protons or gamma rays [2].

## Material and Methods

Cell Lines. We have used two cell lines from human moderately differentiated adenocarcinomas, namely endometrial HEC1B and mammary MCF-7 cells. While both these lines express a functioning estrogen receptor, HEC1B cells are $p53^-$ and MCF-7 $p53^+$. Furthermore the MCF-7 are Caspase 3 negative and rather insensitive to apoptotic stimuli. Normally 1.5 x $10^6$ cells (well below the confluence) were embarked on Stratospheric Balloon in 25 $cm^2$ flasks and thermostated at37°C. Twenty eight flasks of HEC1B or MCF-7 cells have been used during in both BIRBA Missions.

Dosimetry. In both missions qualitative and quantitative measurements of radiation have been obtained by Dr. A. Zanini (INFN – Turin, Italy). The data are reported in the following Table.

| Radiation type | Without Shield | | With Shield | |
|---|---|---|---|---|
| Mission | BIRBA I | BIRBA II | BIRBA I | BIRBA II |
| p | 2.5μSv/h | 2.5 μSv/h | 1.2μSv/h | 1.0 μSv/h |
| n (0-10keV) | 0,1 | 0,04 | 0,05 | 0,02 |
| n (10keV20MeV) | 0,88 | 4,15 | 0,66 | 2,20 |
| n (E >20MeV) | 0.40 | 0.50 | 0.41 | 0.50 |
| n tot | 1,38 | 4,69 | 1,12 | 2.72 |
| a | 1.05 | 2.20 | 1.06 | 1.20 |
| HZE | 6,2 | 6,5 | 2,50 | 1,50 |
| other | 0,1 | 0,5 | 0,02 | 0,09 |
| TOTALE | 11.23 | 16.39 | 5,44 | 6.51 |

BIRBA I Mission (2000).
Twenty-eight flasks were arranged in 4 stacks of 7. Fourteen flasks were loaded in a special radiation-shielded container, fourteen were loaded in a similar container without the radiation shield. Both containers, built by Kayser Italia (Livorno), were thermostated at37°C. The Stratospheric Balloon was launched from Milo (Trapani) and was recovered with major problems in Spain. After landing flasks were recovered, brought in a local laboratory and put in a $CO_2$ incubator overnight. Next morning cells were sealed into an insulated (but not thermostated) container and sent to Naples where they arrived within 24. All cells arrived safely but those loaded in the unshielded container were not analyzed, since we were made aware of partial temperature control failure during the flight. On the contrary, the temperature control in the shielded container was fine. Since Table 1 indicates that shielding did not wholly prevent the exposure of these cells to radiation, these cells, then, have been further analyzed. The controls were constituted by cells kept in Naples in conditions (except radiation) comparable to which the flying cells have been exposed during the mission (cells number, small temperature changes, continuous mechanical agitation). Protein extracts from exposed cells and unexposed control cells were solubilized in denaturing solvent (0.1% Triton X100 plus proteases inhibitors). Extracts from each two flasks (for a total of 7 samples plus controls) were analyzed by Western Blot (see below).

BIRBA II Mission (2002). Experimental set up and procedures.
Even in this case two groups of 14 flasks containing $1.0 \times 10^6$ MCF-7 cells in ~ 60 ml each were embarked into the thermostated Kayser's containers. One container was radiation-shielded. Each group of 14 flasks was, in turn, divided in two stacks of 7 flasks. In each container, seven flasks contained a complete nutrient medium (DMEM + 10% calf serum), seven flasks a medium whose serum was estrogen deprived.
The Stratospheric Balloon was launched from Milo (Trapani) and was recovered without major problems in Spain. All samples were considered suitable for further analysis. Table 1 indicates that during BIRBA II mission the dose of radiation striking the cells was much lower than that caught up in the previous mission. In contrast with the modality adopted during Mission BIRBA I, in this case MCF-7cells were treated in Spain immediately after their recovery with 2 ml/flask of TRIZOL™ , an extremely denaturing and extracting solvent from which is possible to obtain RNA, DNA and proteins at the same time. The TRIZOL extracts arrived to Naples within 24 hs in dry ice. The proteins recovered proteins were finally analyzed by Western Blot.
*Electrophoresis and Western Blots*. The extracts from HEC1B (BIRBA I) and MCF-7 (BIRBA II have been separated on acrylamide gels (10 or 15 %), transferred onto nitrocellulose filters and assayed with specific monoclonal antibodies. All techniques (electrophoresis, blotting, immuno-revelation, scanning of films and normalization) have been performed as previously described [3].
Recent experiences performed in our lab on endometrial cells exposed to artificial radiation (protons, gamma rays), indicated a significant alteration in the expression profile of proteins involved in cell cycle control, apoptosis and response to stress [2]. We now analyze these proteins in cells exposed to natural radiation. It must be underlined that, although the data obtained (in both cell lines) are the expression of averages of many samples, nevertheless they have been obtained by taking advantage of only two flights. This severe limitation must be taken into proper consideration and the Reader should be made aware that any generalization has to be avoided.

## Results and Discussion

BIRBA I mission
The primary antiapoptotic function in the HEC1B cells, is performed by Bcl-XL protein and not by Bcl-2 [3], whose function and phosphorylation status, instead, seems more directly linked to cell cycle control [4]. As already indicated, cells from the shielded container were not totally protected from radiation (Table I). based on this fact, the 40% increase in Bcl-XL expression (as compared to controls (Fig. 1)) could by ascribed to antiapoptotic response of HEC1B cells to radioactive insult. However, this interpretation is disproved by a simultaneous and comparable Bax increase (figure 1). A moderate increase (20-25%) in Cyclin B expression and the marked increase of the stress protein HSP-70 expression are both clearly perceivable in figure 2. The remarkable change in the expression of HSP-70 protein is indicative of an important cellular adaptative response. Similarly relevant is the simultaneous alteration in the phosphorylation status of Cdc-2 and Bcl-2 (fig 3). This particular observation is suggestive of a cell cycle arrest in the G2/M phase. This arrest has been observed in the same cells in separate experiments performed using artificial radiation sources [2]. The ensemble of these experiments in whole, performed on delicate and responsive cells seems to suggest that their exposure to minimal radiation doses, together with the intrinsic mission stresses (mechanical disturbance, minimal but recurrent temperature floating) induces a generalized increase in the expression of all proteins analyzed and may affect cell cycle.
BIRBA II mission
Although the cell lines HEC1B and MCF-7 have in common the estrogen responsivity, the first are far less resistant to dangerous stimuli as compared to MCF-7. These cells, in fact are p53 positive and are Caspase 3 negative, easily trigger DNA self repairing mechanisms and hardly (through alternative pathways) proceed to apoptosis. Finally, as we have shown some time ago [5] within the coding sequence of the *bcl-2* gene, it does exist an Estrogen Responsive Element (ERE). Thanks to the presence of functioning estrogen receptors and the cited ERE in *bcl*-2 gene, MCF-7 cells costitutively over-express Bcl-2 and result naturally resistant to apoptosis. Proteins extracted from MCF-7 exposed to cosmic radiation (see Table 1 and Materials and Methods Section) were separated electrophoretically, transferred on nitrocellulose and analyzed by immuno-chemiluminescent assay (Western Blots). Proteins involved in the induction/resistance of apoptosis, cell cycle regulation and stress were analyzed. The a complete Western Blots panel is presented in Figure 4. Figure 5 reports a quantitative

overview of all protein expressed in various conditions (as marked). This panel allows a direct comparison of the expression changes in cells embarked into an unshielded container (and then exposed in full to cosmic radiation), the cells embarked in the shielded container (only partially exposed) and unexposed controls. The comparison may be extended to cells growing in media containing estrogen or deprived of it. The expression levels in control cells relative to each protein considered in this study has been arbitrarily set to 100. The interpretation of this panel is not straightforward but the capacity of estrogen to modulate the cellular response is quite evident. Once again the simplest conclusion that can be carried out from these data is that the cosmic radiation, even at low doses *but* in association with the intrinsic stresses accompanying the mission, are capable of inducing measurable alterations of gene expression. It is important to underline that all quantitative evaluations performed by densitometry from Western Blots, although obtained by averaging results from seven independent samples (flasks), are expression of a single flying experiment in which the possible causes responsible of observed expression changes, cannot be attributed with absolute confidence to the sole radiation. It is possible that even during a short space mission living cells, exposed to stresses of various types and intensities, have appreciably lower their threshold of sensitivity to radiation. It is also possible that the observed effects originate from a combination of other simultaneous causes most of which have not been yet identified. Perhaps, these observations indicate that experimentation with Stratospheric Balloons, as long as are adequate in number and type, may turn out of great utility in the evaluation of risks to which crew members may be exposed during long lasting space missions and in the establishment of suitable and opportune countermeasures.

## Acknowledgments

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## References

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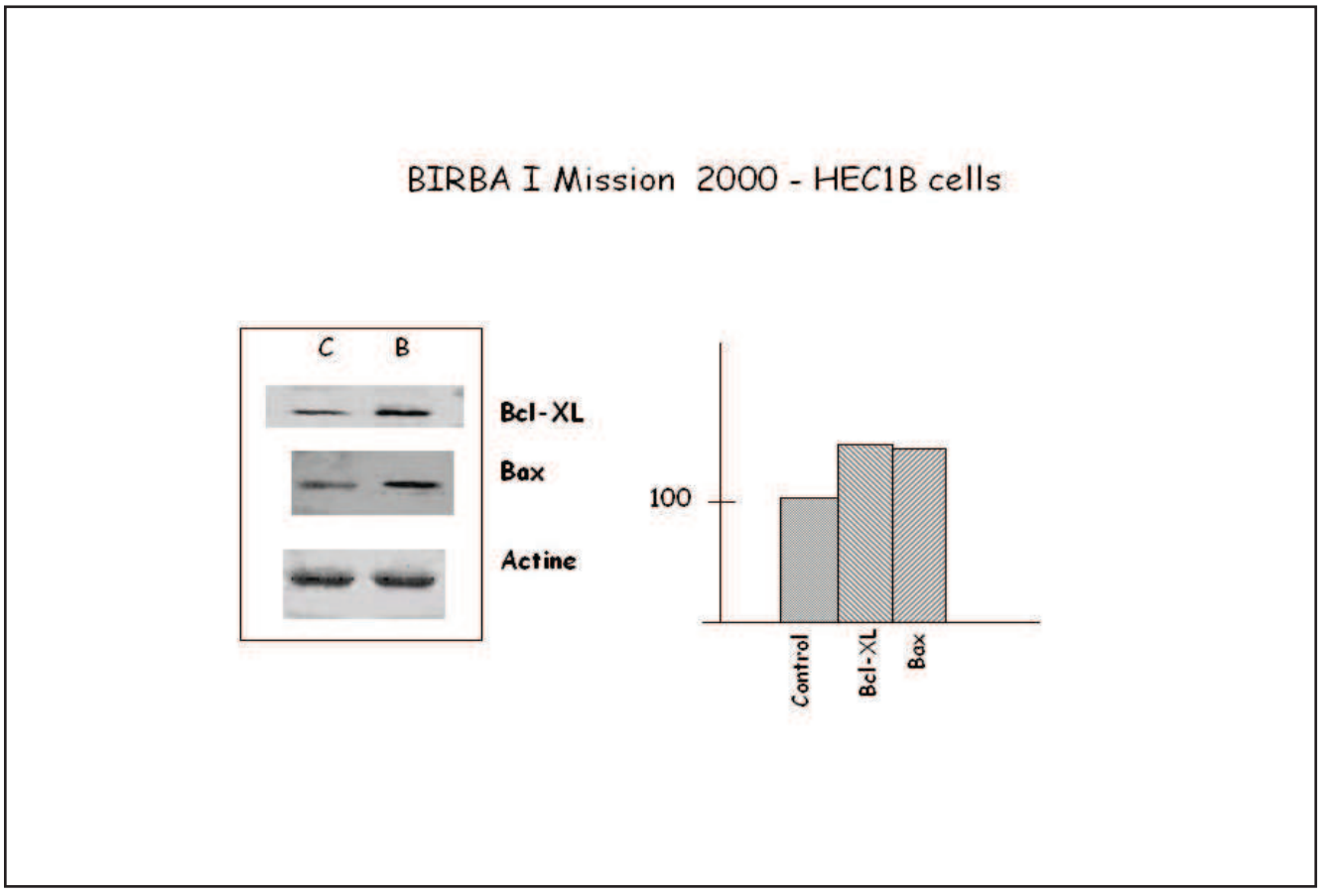


*Fig. 1. The increase in the expression of Bcl-XL is accompanied by a concomitant increase in the expression of Bax. Dasimetry is reported in Table 1*

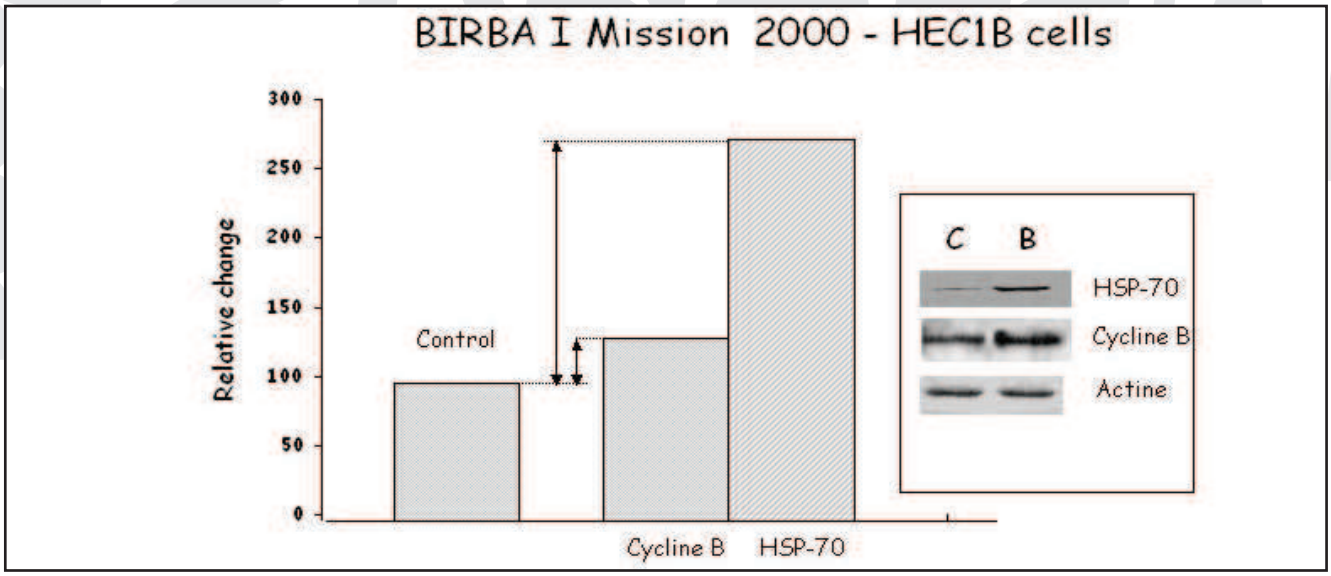


*Fig. 2. HSP 70 and Cyclin B levels in endometrial cells as compared to controls. Dosimetry is reported in Table 1.*

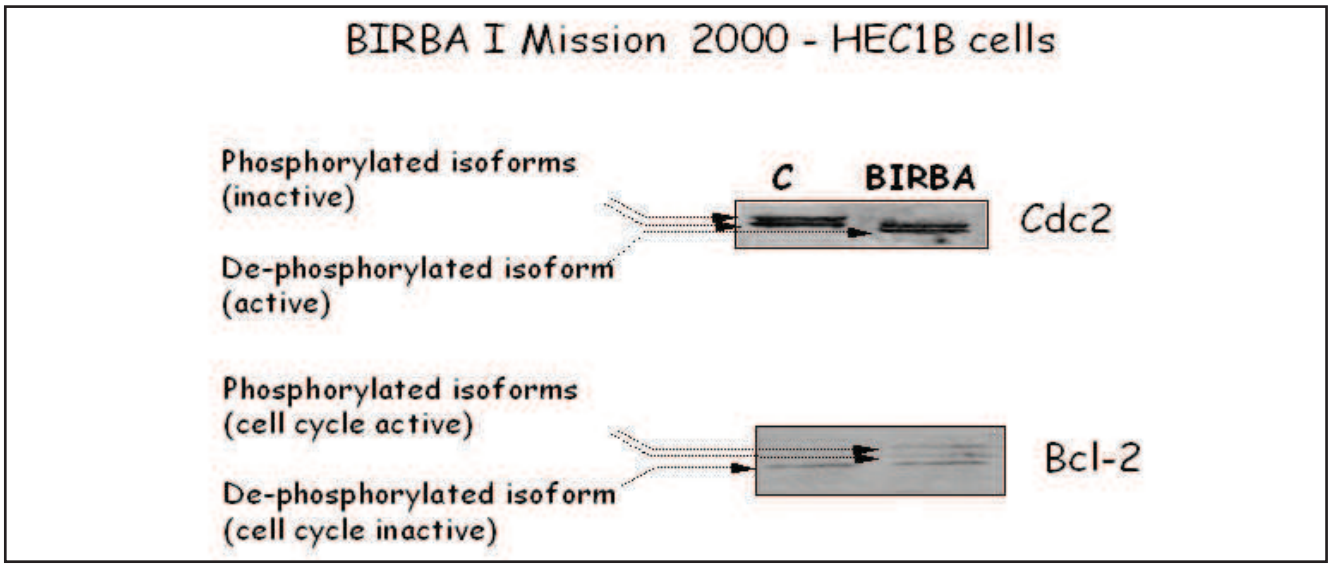


*Fig. 3. Phosphorylation status of Cdc-2 and Bcl-2 in controls (C) and samplesfrom BIRBA (I) exposed to cosmic radiation. Dosimetry is reported in Table 1.*

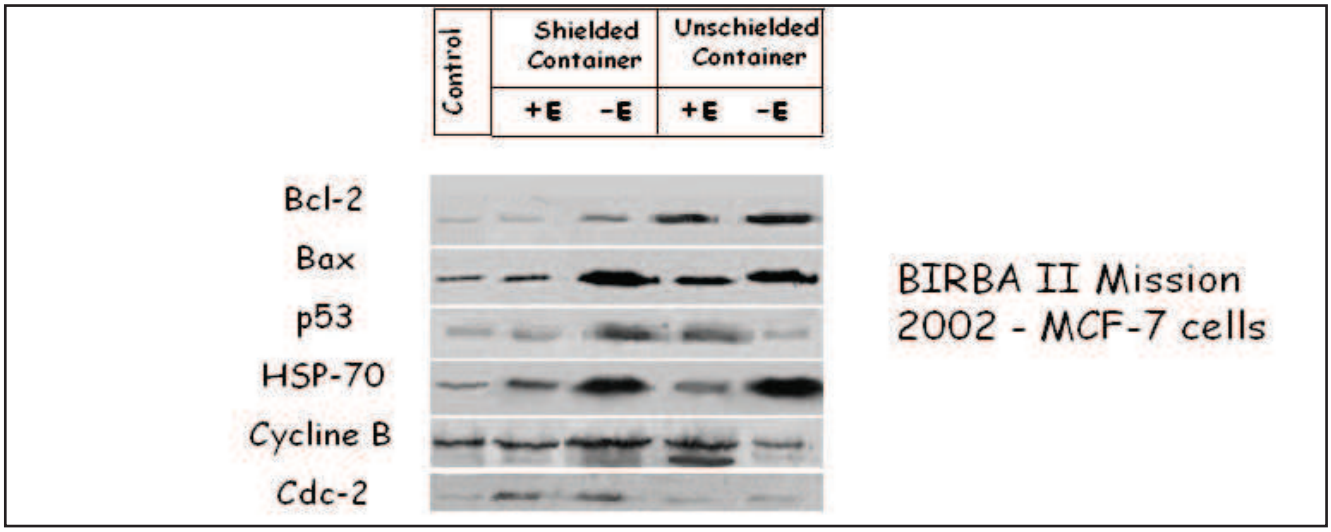


*Fig. 4. Expression levels of some proteins extracted from MCF-7 cells in the presence (+) or absence (-) of Estrogen (E). Dosimetry is reported in Table 1.*

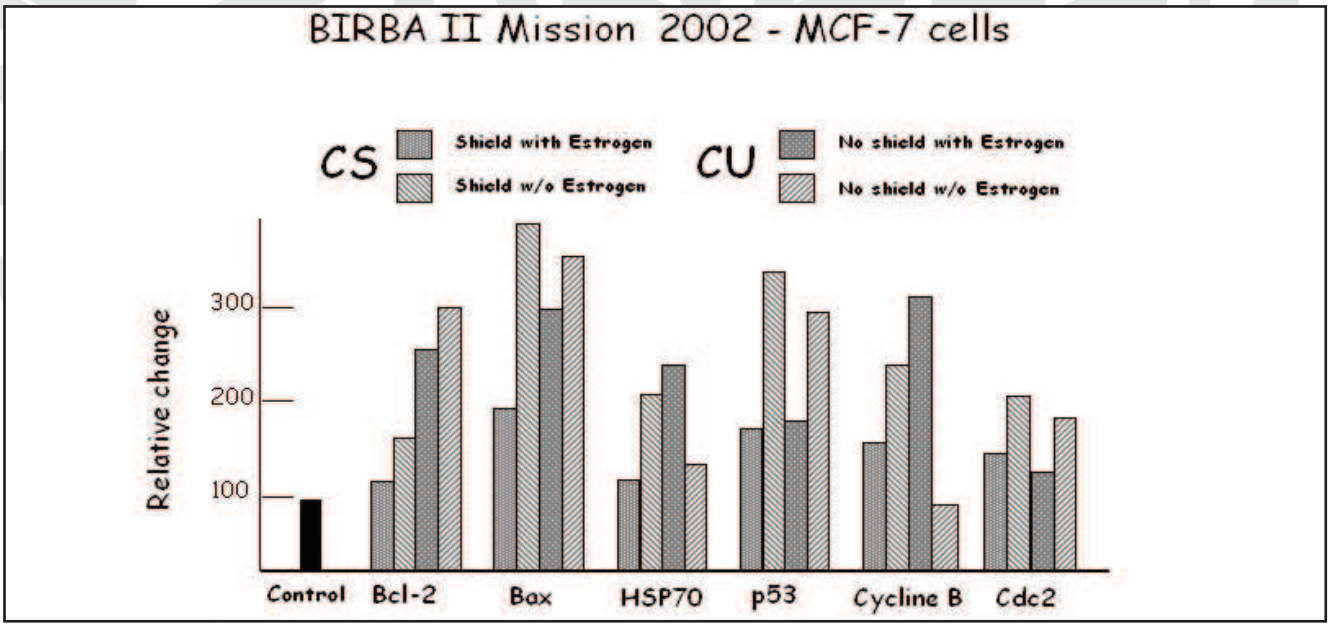


*Fig. 5. Relative changes in the expression of some proteins extracted from MCF-7 cells in the presence (+) or absence (-) of Estrogen (E). Container CS in shielded; container CU is unshielded. Dosimetry is reported in Table 1.*