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# Serum protein concentration in low-dose total body irradiation of normal and malnourished rats

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ABSTRACT

Among the radiotherapeutics' modalities, total body irradiation (TBI) is used as treatment for certain hematological, oncological and immunological diseases. The aim of this study was to evaluate the long-term effects of low-dose TBI on plasma concentration of total protein and albumin using prematurely and undernourished rats as animal model. For this, four groups with 9 animals each were formed: Normal nourished (N); Malnourished (M); Irradiated Normal nourished (IN); Irradiated Malnourished (IM). At the age of 28 days, rats of the IN and IM groups underwent total body gamma irradiation with a source of cobalt-60. Total protein and Albumin in the blood serum was quantified by colorimetry. This research indicates that procedures involving low-dose total body irradiation in children have repercussions in the reduction in body-mass as well as in the plasma levels of total protein and albumin. Our findings reinforce the periodic monitoring of total serum protein and albumin levels as an important tool in long-term follow-up of pediatric patients in treatments associated to total body irradiation.

## 1. Introduction

Among the radiotherapeutics' modalities, total body irradiation (TBI) is used as treatment for certain hematological, oncological and immunological diseases, being an important part of the treatment in bone marrow and stem cell transplants.

For patients at low risk of cell engraftment rejection, non-myeloablative conditioning regime with single fraction low-dose TBI (2–3 Gy) is an effective treatment option, alone or combined with chemotherapy. However, the incidence of complication induced by TBI is elevated, presenting as common problems such as renal toxicity, cataracts and functional reduction of the pituitary gland (Onal et al., 2012). In parallel, another aggravating factor of complications that arises from the application of TBI is the general health of the patients which, in most cases, present weight loss due to the simultaneous occurrence of malnutrition (Oliveira, 2007; Ollenschlager et al., 1991).

From animal studies, it has been observed that malnutrition interferes directly on growth and body development, especially on body mass, triggering a sequence of adaptation mechanisms, especially on the musculoskeletal system, such as changing the fiber type (Paiva et al., 2012; Toscano et al., 2008, 2010).

The plasma concentration of total protein is reported as an important and reliable parameter for malnutrition assessment (Nwokocha et al., 2012a; Santos et al., 2004), although clinically, the isolated dosage of total protein has little value, since a change in one of their fractions (globulins or albumin) can be compensated by changing the other one, where 6.4 g/dL is accepted as the minimum threshold (Labtest, 2014).

The albumin level is one of the several clinical parameters of general health as well as the most widely used biochemical indicator of malnutrition. There is a strong correlation between lower-than-normal albumin levels and the incidence of morbidity and mortality in hospitalized patients. Serum values lower than 3.5 g/dL are considered indicative of malnutrition, chronic liver diseases, nephrotic syndrome and of state of protein catabolism (Doweiko and Nompleggi, 1991; Fontoura et al., 2006; Vannucchi et al., 1996).

In this context, taking into account the importance in the follow-up of pediatric patients after TBI treatment, the aim of this study was to evaluate the long-term effects of low-dose total body irradiation on plasma concentration of total protein and albumin using prematurely and undernourished rats as animal model.

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**Table 1**
Composition of the experimental diets.

| Ingredients | Control diet (17% protein) | Hypoproteic diet (8% protein) |
|---|---|---|
| | g/kg | g/kg |
| Casein (84%)[a] | 202.40 | 95.23 |
| Corn starch | 527.03 | 634.26 |
| Sucrose | 100.00 | 100.00 |
| Soy oil | 77.00 | 77.00 |
| Fibers | 50.00 | 50.00 |
| Vitaminic Mix 1 | 10.00 | 10.00 |
| Mineral Mix 2 | 35.00 | 35.00 |
| Bitartrate Choline | 2.50 | 2.50 |
| L-methionine | 3.00 | 3.00 |
| Butylated hydroxy toluene (BHT) | 0.01 | 0.01 |
| Total | 1006.94 | 1007.00 |

Values obtains from AIN-93G, Reeves et al. (1993).
[a] Casein showed 84% purity (84 g protein per 100 g of casein).

## 2. Materials and methods

This study was approved by the Ethics Committee on Animal Use of the Biological Sciences Center of the Federal University of Pernambuco (CEUA-UFPE), under the case No. 23076.021454/2013-69.

The protocols used are in accordance with the guidelines suggested by the Brazilian College for Animal Experimentation, and with the international standards established by the National Institute of Health Guide for Care and Use of Laboratory Animals.

### *2.1. Experimental groups and diets*

This study employed normal and undernourished Wistar rats lineage (Bento-Santos et al., 2012), which were kept in an environment with a temperature of 23 °C (±2) in constant light cycles (6 a.m.–6 p.m.) and darkness (6 p.m.–6 a.m.).

120-day-old virgin rats were selected and then put to mate (2 females/1 male). It was designated as the day of conception (day 1 of pregnancy) the one in which it was observed spermatozoa through the vaginal smear.

Pregnant rats were transferred to individual maternity cages, where it received filtered water and normoproteic controlled diet (17% of protein) in accordance with the recommendations of the AIN-93G (Reeves et al., 1993) or a hypoproteic and isocaloric diet (8% of protein) throughout the whole pregnancy and lactation (21 postnatal days), in an *ad libitum* regime.

The ingredients used for the preparation of the diets were provided by Rhoster Indústria e Comércio Ltda (Table 1).

After 24 h birth, the litters were limited to eight pups for each mother. At weaning, the rats-mothers and their daughters were sacrificed, while the male offspring started receiving the standard normoproteic diet (Presence Rats and Mice™), also in an *ad libitum* regime.

To establish the experimental groups ensuring heterogeneity, the animals were randomized and groups were composed of 3 litters from different parents, totalizing and 9 animals. Then, four groups with 9 animals each were formed, namely:

Normal nourished (N): fed with a diet of 17% of protein during the periods of pregnancy and lactation (diet offered to the pregnant lactating rats). After weaning, the animals received standard rodent diet.

Malnourished (M): fed with a diet of 8% of protein during the periods of pregnancy and lactation (diet offered to the pregnant lactating rats). After weaning, the animals received standard rodent diet.

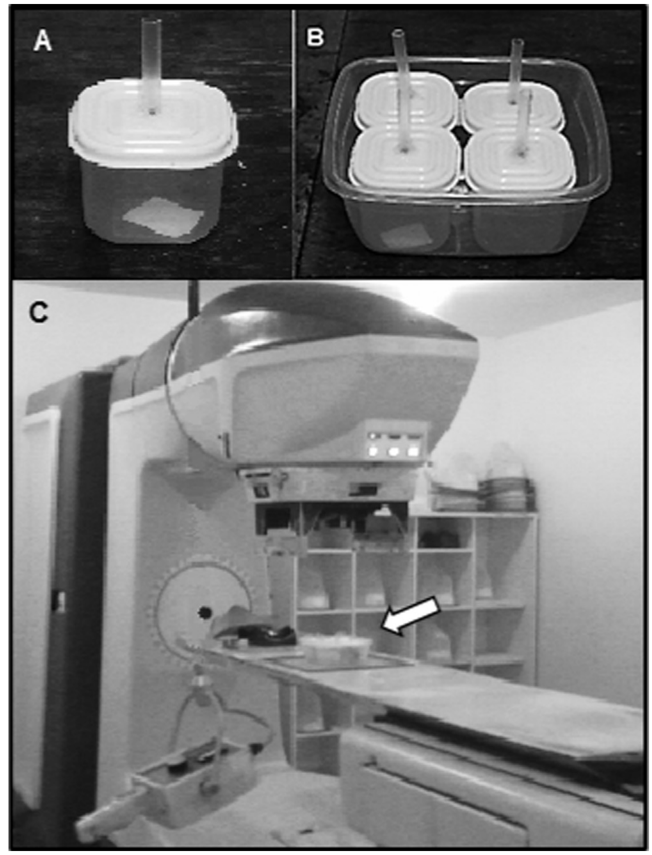


**Fig. 1.** (A) Plastic holding device for one rat; (B) plastic container with four holding devices; (C) setup of irradiation ($^{60}$Co source) showing the container (arrow).

Irradiated Normal nourished(IN): fed with a diet of 17% of protein during the periods of pregnancy and lactation (diet offered to the pregnant lactating rats). After weaning, the animals received standard rodent diet.

Irradiated Malnourished (IM): fed with a diet of 8% of protein during the periods of pregnancy and lactation (diet offered to the pregnant lactating rats). After weaning, the animals received standard rodent diet.

Every 10 days the body mass of the animals were recorded until the age of 90 days, this is about equivalent to 9-year-old child (Quinn, 2005; Sengupta, 2013), when they were sacrificed by decapitation, and the blood was collected immediately in test tubes and centrifuged for 20 min at 1482 rcf (3500 rpm), at 4 °C. The supernatant serum was separated and placed in polypropylene microtubes with a capacity of 1,5 ml and frozen at −80 °C for subsequent quantitation.

### *2.2. Irradiation*

At the age of 28 days (equivalent to approximately 2-year-old child), rats of the IN and IM groups underwent total body gamma irradiation with a source of cobalt-60 ($^{60}$Co) (Theratron780 irradiator – AECL/Best Medical), 1.25 MeV energy (dose-rate of 75 cGy/min), receiving 2.5 Gy in a single section. For rats, this is non-myeloablative dose as for humans (Duran-Struuck and Dysko, 2009).

In order to prevent movements during the irradiation, the animals were placed in individual restraint plastic containers, specifically developed for this purpose. During the irradiation process, 4 rats were exposed simultaneously. For this, four individual containers were placed into a lager plastic box, having external dimensions 17.0×17.0×6.5 cm as shown in the Fig. 1.

At that moment, each rat weighed about 50 or 80 g, corresponding

**Table 2**
Mean values of body mass gain in the period between 30 and 90 days of age.

| Days | Body mass (g) | | | |
|---|---|---|---|---|
| | Groups (n=9) | | | |
| | N | M | IN | IM |
| 30 | 80.42 ± 1.87 | 53.90 ± 1.19[a,b] | 73.83 ± 2.42 | 48.06 ± 1.24[a,b] |
| 40 | 148.60 ± 3.09 | 116.50 ± 2.64 | 131.10 ± 3.83 | 117.06 ± 6.21 |
| 50 | 195.50 ± 10.36 | 188.50 ± 10.11 | 180.89 ± 4.90 | 161.06 ± 5.74 |
| 60 | 244.90 ± 10.26 | 238.80 ± 5.82 | 235.28 ± 5.46 | 225.44 ± 5.85 |
| 70 | 279.17 ± 15.41 | 270.10 ± 4.76 | 256.56 ± 3.63 | 230.67 ± 5.65 |
| 80 | 308.62 ± 19.35 | 290.50 ± 6.44 | 282.78 ± 3.88 | 240.39 ± 6.77 |
| 90 | 322.77 ± 7.62 | 296.15 ± 5.82[a,c] | 296.78 ± 7.93[a,c] | 247.67 ± 7.84[a] |

Data are presented as the mean ± SEM obtained from 9 rats of different litters in each experimental group. N normo-nourished group; M malnourished group; IN irradiated normo-nourished group; IM irradiated malnourished group; n number of animals. a significant difference $p < 0.05$ in relation to N. b Significant difference $p < 0.05$ in relation at IN. c Significant difference $p < 0.05$ in relation to IM.

respectively to the average weight of a malnourished or eutrophic animal. To ensure the uniformity of irradiation, the box was filled with water.

### *2.3. Quantification of proteins*

For the quantification of total protein in the blood serum, colorimetry was used by the Biuret method in accordance with the LABTEST (Brazil) test protocol, reference 99/250, batch (3004) and linearity between 1.0 and 14.0 g/dL (Labtest, 2014), maintaining the usage proportions for polystyrene 96 wells culture plate with a flat bottom. The readings of the absorption band at 545 nm for each animal (in triplicate) were performed using a spectrophotometer (Fluostar Omega/BMG Labtech, Germany). From the triplicate, an arithmetic average of the two closest values was performed to determine the protein concentration of each animal.

Albumin was also quantified by colorimetry (bromocresol green) in accordance with the LABTEST (Brazil) test protocol, reference 19/250, batch (4004) and linearity up to 6.0 g/dL (Labtest, 2014). The usage proportions in a polystyrene 96 wells culture plate with a flat bottom were maintained. The readings of the absorption band, also in triplicate for each animal, were taken at 630 nm using the same spectrophotometer. The arithmetic average of the two closest values of the three obtained was also used for determining the albumin concentration of each animal.

## 3. Statistical analyses

The data were subjected to homogeneity and normality tests. A one-way analysis of variance (ANOVA) was used to compare the different treated groups. Post-hoc analyses were performed using multiple comparison test of *Tukey* $p < 0.05$ values were considered significant to indicate differences. The tests were performed using the GraphPadPrism version 6.0 for Windows (GraphPad Software Inc. San Diego/CA, EUA).

## 4. Results

Table 2 shows the average of weight values related to body mass gain. Body mass were obtained at an every 10 days interval, in the period between the 30th and 90th day of life.

Fig. 2 contrasts the mean values of body weights after 30, 60, and 90 days of age.

Fig. 3 shows the evolution of body-mass gain for N and IN groups fed a normal protein diet during pregnancy and lactation.

Fig. 4 shows the evolution of body-mass gain for groups M and IM, which were fed a hypoproteic and isocaloric diet.

Table 3 shows the concentrations of total protein and albumin in blood serum at the age of 90 days, which were obtained from 9 rats of different litters in each experimental group. The data are presented as the mean and standard error of mean.

Fig. 5 shows the behavior of levels of serum albumin and total protein in the four experimental groups.

## 5. Discussion

In this work, the general health conditions of pediatric patients who underwent low-dose total body irradiation in childhood were simulated from whole body irradiation of prematurely and undernourished rats as animal model (28-day-old rat is about equivalent to 2-year-old child).

The composition of the purified isocaloric low-protein diet (8% of protein), employed in this work, was formulated from AIN-93G (Reeves et al., 1993), which has been recommended by the American Institute of Nutrition in studies aiming to reproduce in rodents the same conditions of metabolic changes resulting from malnutrition.

Several researches have adopted this low-protein diet to induce malnutrition in rats and, as established by International Society Rhenal Nutrition and Metabolism (ISRNM), and the survey of undernutrition is conducted by the evaluation of the body-mass gain (Alheiros-Lira et al., 2015; de Brito Alves et al., 2014; de Santana Muniz et al., 2013; Falcão-Tebas et al., 2012; Ferro Cavalcante et al., 2016; Latorraca et al., 1998; Neiva et al., 1999). Another criterion also adopted by ISRNM as biochemical parameter is the measurement of the concentration of serum albumin (Vegine et al., 2011).

The follow-up study has been sought to investigate changes in the plasma concentrations of total protein and albumin in normal and undernourished rats during pregnancy and lactation, after an acute whole-body exposure, delivering 2.5 Gy from low linear energy transfer (LET) radiation source ($^{60}$Co), using the setup of irradiation presented in the Fig. 1.

Similar to humans, the metabolic status of the animal tissues is well represented by the blood plasma composition, being one of the ways of evaluating disorders in the functioning of organs and specific metabolic imbalances or of nutritional origin, besides offering support in the interpretation of liver, renal, pancreatic, bone and muscle function. However the interpretation of the biochemical profile is complex due to the mechanisms that control blood metabolites as well as factors related to age, race, climate and stress (González and Scheffer, 2002).

Table 2, which is graphically represented in the Fig. 2, reveals that animals belonging to the Malnourished (M) and Irradiated Malnourished (IM) groups showed significant differences regarding body mass gain in 30 days ($p < 0.05$), when compared to the Normal (N) one. These data corroborate with studies that have used this type of intervention. Like it was well reported in the literature, undernourished rats during pregnancy and lactation have low birth weight (de Brito Alves et al., 2014; Falcão-Tebas et al., 2012; Neiva et al., 1999).

In the Fig. 2, considering 90-day-old rats (about 9-year-old equivalent human age) and taking to the normal group as reference, one can observe a reduction in body-mass gain for all other groups, namely M, IM and Irradiated Normal (IN), which the groups M and IN lost 8% and IM lost 23%. Taking into account IN group, the combination of irradiation and malnutrition has significant effect on body-mass loss throughout this life-span, as pointed out by the IM group. As also can be observed by comparing the groups M and IN, either malnutrition or irradiation, acting separately, impact similarly on body-mass loss (Mucerino et al., 1995).

Figs. 3 and 4 compare the influence of the radiation in the body-mass gain, considering groups with the same diet. The results indicated that low-dose TBI negatively impacted in the evolution of body-mass gain.

Returning to animals at 90 days of life, that underwent only

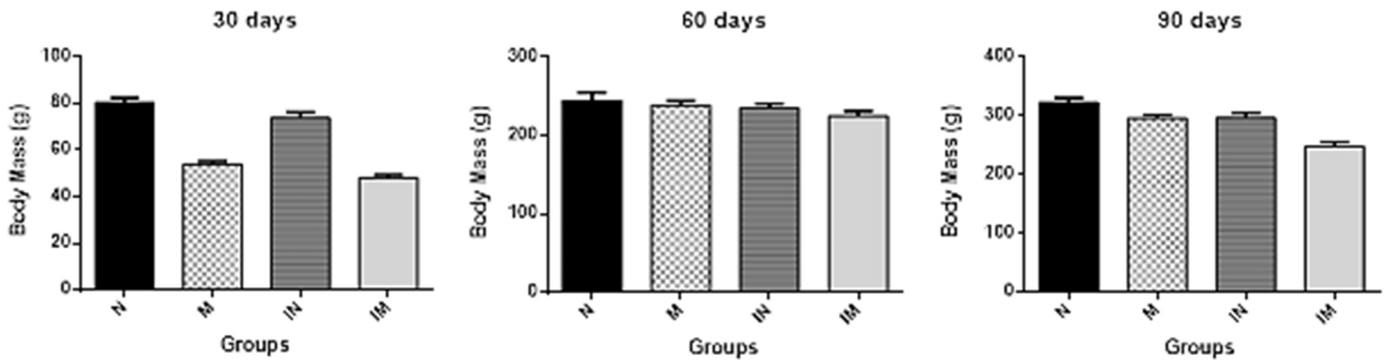


**Fig. 2.** Mean values of body mass at 30, 60 and 90 days of life for the four experimental groups. Data are presented as the mean ± SEM obtained from 9 rats of different litters in each experimental group $p < 0.05$.

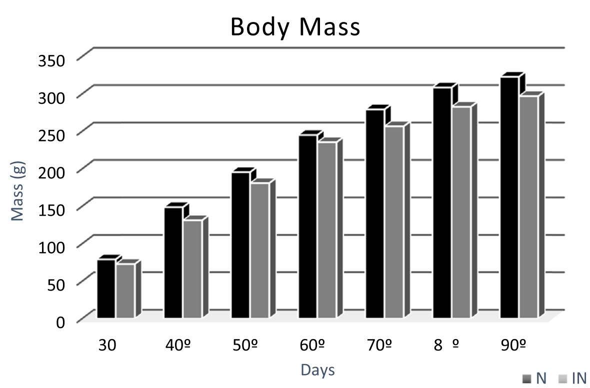


**Fig. 3.** Body mass gain of the animals belonging to the groups N and IN in the period between 30 and 90 days of age.

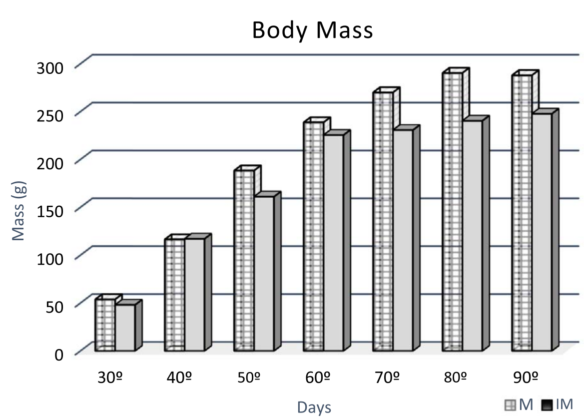


**Fig. 4.** Monitoring of body mass gain of the animals belonging to groups M and IM for the period between 30 and 90 days old.

**Table 3**
Average values of total protein and plasma albumin at 90 days of age.

| Groups n=9 | Total protein (g/dL) | Albumin (g/dL) |
|---|---|---|
| N | 8.06 ± 0.42 | 5.35 ± 0.43 |
| M | 7.37 ± 0.51 | 5.07 ± 0.21 |
| IN | 5.08 ± 0.24$^{a,b}$ | 3.68 ± 0.28$^{a,b}$ |
| IM | 4.93 ± 0.40$^{a,b}$ | 3.62 ± 0.35$^{a,b}$ |

N normal-nourished group; M malnourished group; IN irradiated normo-nourished group; IM irradiated malnourished group; a significant difference $p < 0.05$ in relation to N; b Significant difference $p < 0.05$ in relation to M.

malnutrition during pregnancy and lactation, the results present in the Table 3 showed no differences in the concentrations of albumin in the blood serum relative to the control group. Statically summarized in the Fig. 5, these results are in accordance with de Brito Alves et al. (2014), who showed that malnutrition has little or no impact on the serum concentrations of albumin and total protein in the long-term. However, the process of irradiation leads to low levels of plasma concentration of total protein and albumin, suggesting that TBI causes a decrease in protein synthesis, which lasted long-term. In parallel, malnutrition causes a decrease in albumin synthesis too. As the most abundant plasma protein, albumin has an important role in maintaining the circulating plasma volume, being responsible for the coloidosmotic pressure (Santos et al., 2004; Whicher and Spence, 1987).

Other studies with whole irradiated rats also observed that the level of total protein and albumin in blood serum display a decrease in their concentrations (Nwokocha et al., 2012a, 2012b; Vasile et al., 2009; Wang et al., 2015). However, those works did not observe the behavior of these concentrations for a period as long as the one employed by Moulder et al. (2004) that is similarly to the ours.

Moulder et al. (2004) found that a dose of 17 Gy radiation, with a dose-rate of 1.73 Gy/min, induced proteinuria associated with low levels of total protein and albumin, and the animals developed renal failure after 38 weeks of exposure. However, the dose used in our study, 2.5 Gy (0.75 Gy/min), was 6.8 times lower, indicating that low doses of radiation are also able to interfere permanently in the protein synthesis. Rats, dogs, pigs and primates exhibit physiological and histological changes similar to those observed in persons with renal lesion induced by radiation, although the time between irradiation and development of kidney disease varies occurring earlier than in humans (Augustine et al., 2005).

Another interesting result of our research is associated to the differences found in the evolution of the plasmatic concentrations of total protein and albumin between IN and M groups, in 90 days. Comparing the two groups, although no differences in body-mass were found, the decrease in the concentration of the serum proteins and albumin is directly linked to the total body irradiation.

Rats are the most employed *animals* for experiments, corresponding to approximately 20% of the total number of mammals used for scientific purposes, especially for *in vivo* studies (Sengupta, 2013). Using this animal model, our results seem to be in good agreement with the known long-term manifestation of malfunction of liver and kidney after acute total radiation in humans (IAEA, 2010), since abnormal serum albumin levels may indicate that kidneys or liver isn't working correctly.

Besides factors such as the type of radiation, absorbed dose and dose-rate, side effects induced by ionizing radiation strongly depend on the genetic and epigenetic history, which directly reflect on the individual radiosensitivity. In this sense, there are populations with genotypes that induce radiosensitivity that may be at increased risk of developing cancer with low-dose exposures than the general public (Augustine et al., 2005). In our work, this inter-individual genetic aspect was taken into account by the employment of three different families of rats, while the nutrition condition was the unique epigenetic

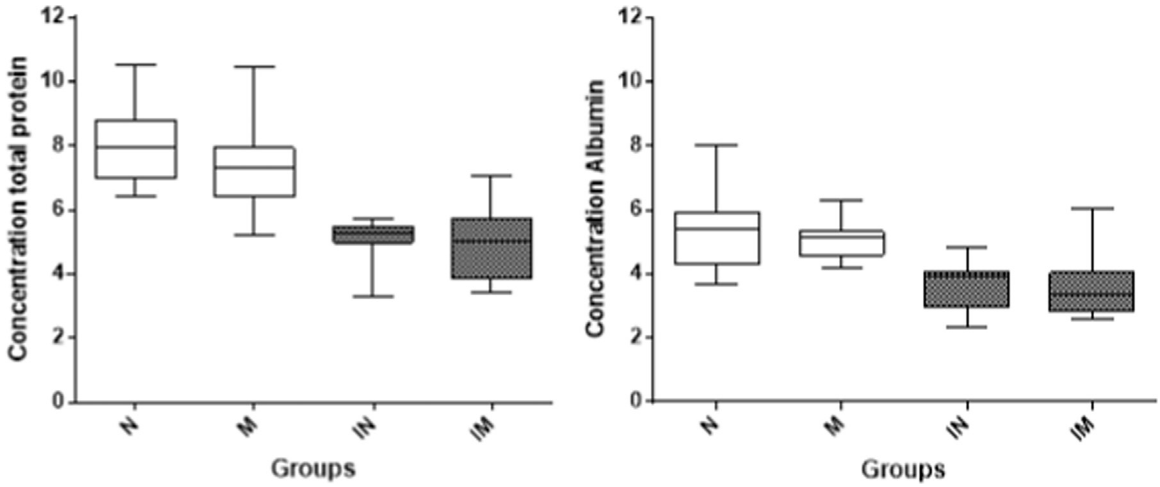


**Fig. 5.** Boxplots related the quantity of albumin and total protein found in serum of the four experimental groups.

factor considered in this research.

## 6. Conclusions

Despite the need for further studies considering different doses and ages, this research indicates that procedures involving low-dose total body irradiation in children have repercussions in the reduction in body-mass as well as in the plasma levels of total protein and albumin, even at dose levels below the conventionally used for TBI. Based on animal model, our findings reinforce the periodic monitoring of total serum protein and albumin levels as an important tool in long-term follow-up of pediatric patients in treatments associated to total body irradiation.

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