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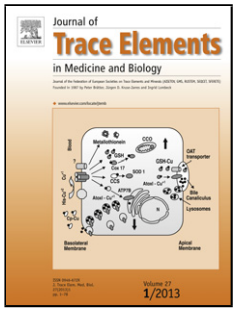

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^{67}Ga as a biosensor of iron needs in different organs. Study performed on male and female rats subjected to iron deficiency and exercise.

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Abstract

The aim of the present study was to determine the iron needs in different organs and tissues using ^{67}Ga as a biosensor in males and females rats subjected to iron deficiency (ID) and voluntary exercise (EX). ^{67}Ga citrate was injected i.p. to female and male Wistar rats (n=5/sex/group). Groups: Control (sedentary conditions), Control+EX, ID and ID+EX. To determine the ^{67}Ga uptake, samples from the following regions of interest (ROIs) were extracted 12h post-injection: blood, liver, gonads, bone marrow, heart, adrenal glands, skeletal muscle, stomach, kidney, eyeball, sciatic nerve, small intestine and peritoneum. The total ^{67}Ga uptake was 412% higher in ID subjects than in control subjects, being

1011% higher in ID-males than ID-females. In ID-females, the ROIs with the greater ^{67}Ga uptake were blood, kidney and bone marrow, while in ID-males they were sciatic nerve, eyeball and adrenals, which demonstrates that the biodistribution differed between sexes in sedentary conditions but when subjected to EX, the biodistribution was similar in each sex group although females had a greater ^{67}Ga uptake. In ID+EX subjects, the ROIs that showed the highest uptake were sciatic nerve, eyeball and adrenal glands. Using ^{67}Ga as a biosensor, it is possible to identify the needs of iron that each organ requires to perform their functions in normal physiological conditions. In addition, a higher or lower ^{67}Ga uptake in a specific organ may indicate its malfunction or show damage.

Key words: ^{67}Ga, biodistribution, radiophamacokinetics, iron deficiency, exercise, environmental enrichment.

1. Introduction

Iron is a trace element required for oxygen transportation and enzyme systems that regulate redox balance, cellular respiration and proliferation. In the plasma, it is distributed by forming bonds with different proteins; hemoglobin, transferrin, hemosiderin, lactoferrin and albumin

[1,2].

Iron distribution depends on the metabolic needs of each tissue and its ability to capture, store or transfer this trace element [3]. Iron deficiency is considered the most prevalent type of malnutrition, which may or may not course with anaemia [4]. The biochemical

alterations linked to iron deficiency may lead to clinical manifestations, which are often underdiagnosed, e.g. motor, cognitive, behavioral and degenerative disorders [5,6]. Daily iron requirements are specific for different stages of life, the sex of the individual and certain conditions that increase its demand, such as pregnancy or exercise [7,8]. Regarding sex, it is known that iron homeostasis may be modulated differently by estrogen and testosterone [9,10]. Voluntary exercise (motor activity not required for survival or homeostasis and that is not conditioned by some external factor) and forced exercise, optimize the absorption and biodistribution of iron [11] and increase bone marrow heme biosynthesis [12,13]. In normal conditions, tissue iron absorption is specifically increased during periods of cellular differentiation, tissue remodeling and growth (organogenesis, ontogenesis), furthermore, iron demands are also higher in neoplasms [14,15]. In addition, under iron deficiency conditions, homeostatic changes lead to the optimization of physiological needs of each organ and tissue (e.g. increased desaturation and number of iron transport proteins and an increase of transferrin receptors (TfR)). Hence, its tissue distribution depends on the needs, sex and physical conditions (such as exercise).

^{67}Ga is a radionuclide [16] that has been used for almost 50 years in nuclear medicine imaging as a radioactive tracer for the diagnosis and monitoring of pathological processes in inflammatory conditions [17,18] such as cancer [19,20], metastasis [21,22], infections [23,24] and autoimmune diseases [25,26]. The chemical properties of gallium allow it's binding to transport and fixing iron proteins, which makes it possible to consider it as an iron analog [27]. Ga and Fe present similar ionic radii (0.620-0.645 Angstroms), which gives Ga the ability to make covalent bonds with proteins responsible for Fe3 transportation and metabolism such as: transferrin (Tf), lactoferrin, and albumin [28]; subsequently it is captured at the tissular level by TfR for its internalization by receptor-mediated endocytosis

[29]. Currently, the potential of ^{67}Ga as a tracer and its interaction with iron binding proteins and consequent tissue localization has not been evaluated. Therefore, the aim of this work is to determined and quantify the iron requirements in different regions of interest (ROIs) in Wistar rats depending on sex and exercise when subjected to iron deficiency, using ^{67}Ga as a biosensor.

2. Materials and methods

2.1.Ethics statement

All studies were conducted in accordance with approved institutional protocols in agreement with the Principles and Procedures described by the National Institute of Health, Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, in accordance with the Local Ethics Committee. In order to obtain samples, the test subjects were euthanized with CO_2 to minimize suffering.

2.2. **Animals and diet**

Wistar rats were maintained under standard vivarium conditions: a 12:12 light/dark cycle (light was turned on at 5 o'clock) was used, controlled temperature of about 22 ± 2°C with free access to food and water.

The study was conducted using pup rats, whereas their parents were subjected to the following conditions: 14 days prior to their mating period, 10 female rats (3 months old or 250 g) were placed under an iron deficient diet (10 ppm FeSO4, Lab Diets AIN-76W / 10). Two other rats received a control diet (100 ppm FeSO4, Lab diets AIN-76W / 100). During pregnancy up until the 70th postnatal day (PND) the corresponding diets were maintained.

As of weaning (PND 21) pups were randomly separated by sex, classified per type of diet and were given access to voluntary exercise (EX) through environmental enrichment from this period (n = 3 rats / box) until the 70th PND in the following groups: a) Control, b) Control+EX, c) Iron deficiency and d) Iron deficiency+EX. 7 females and 7 males were used in each group.

2.3. Voluntary exercise (EX) – Environmental Enrichment

As of the 21st until the 70th PND, rats were randomly assigned to groups with environmental enrichment and placed inside boxes according to their sex (90cmX40cmX60cm), which were equipped with a running wheel (diameter: 20 cm, width: 12 cm) to perform voluntary exercise. The position of PVC tunnels and toys (small balls, plastic figures and a metal wire bridge) were changed daily.

2.4. ^{67}Ga uptake activity: ROI´s quantification

Considering that 1Bq = 1 disintegration/second, 37 MBq of ^{67}Ga in 0.4 mL were administered to 15 rats on the 70th PND. It is worth mentioning that the exposure is negligible because the administered activity is a trace dose. Three rats were euthanized every 2, 4, 8, 12 and 24 hours in a CO_2 chamber with a gradual release of O_2 at a rate of 0.5L CO_2/min/10 min. Afterwards, the following ROIs were extracted: blood (intracardiac puncture), liver, gonads, bone marrow, heart, adrenal glands, skeletal muscle, stomach, kidney, eyeball, sciatic nerve, small intestine and peritoneum. ^{67}Ga activity was measured in each ROI with a dose calibrator (Capintec Inc, CRC-55tR). The time of maximum activity (t_{max}) [(MBq/g) / MBq] was determined in each ROI; results obtained were

normalized by measuring the activity of the administered quantity, mass of the ROI and radioactive decay.

The uptake was compared between sexes and the different groups.

For the statistical analysis, the results were presented as means and standard deviations (mean±SD) to show differences between all groups.

2.5. Iron bound to hemoglobin (Fe-Hb)

To demonstrate the presence of ID, the levels of Fe-Hb were indirectly measured in each study group employing the following formula [30]:

$$Fe - Hb(mg) = \frac{\left(\left(\frac{Hb}{L}\right) * (body\ weight) * 6.7 * 0.335\right)}{10000} \qquad eq.1$$

Where Hb (g) contains 0.335% iron. The blood volume in growing rats is 6.7% of body weight (g).

To determine Hb, 1 ml of blood was taken from the studied subjects; it´s concentration was determined in triplicate by the cyanomethemoglobin method using Drabkin solution (Randox Mexico SA de CV) [31].

SPSS 21® software was used for statistical analysis; a descriptive statistical analysis was initially performed for each variable that was studied. The results are presented as means and standard deviations (mean±SD). As distribution was nonparametric, the data were statistically analyzed using a Mann Whitney *U* tests to compare the differences between two groups (eg “ID” and “C” groups, or male and female groups). Outcomes of $p<0.05$ were considered statistically significant results, with a confidence interval of 95%.

3. Results

3.1. ^{67}Ga uptake by each ROI

The maximum activity was established at t_{max}=12 h. Therefore, experimental groups were euthanized at that time after ^{67}Ga administration.

The n was reduced to 10 subjects per group (5 females and 5 males) due to a lack of activity in some rats at t_{max}, probably caused by an increase of the basal metabolism of the rats which could have caused a rapid clearance of ^{67}Ga.

The results show that in the ID group ^{67}Ga uptake was 412% higher than in the control group. Greater activity was registered in the ID group when compared to the control group in the following ROIs: eyeball (18720%), sciatic nerve (13082%), adrenal (4138%), liver (360%), heart (198%), small intestine (159%), muscle (146%), stomach (135%), bone marrow (127%), gonads (93%), kidneys (67%) and peritoneum (34%); as shown in Figure 1.

3.2. **Exercise effect on ^{67}Ga uptake for each ROI**

It was found that EX increases the uptake of ^{67}Ga by 20% in control+EX and 155% in ID+EX groups. Furthermore, ^{67}Ga uptake was 1259% higher in ID+EX when compared to control+EX. In addition, the uptake was increased for ID+EX when compared to control+EX in each of the following ROIs: sciatic nerve (16548%), adrenals (5043%), eyeball (4222%), small intestine (482%), heart (345%), gonads (331%), skeletal muscle (297%), peritoneum (234%), blood (196%), kidney (70%), liver (68%), bone marrow (35%) and stomach (33%), as shown in Figure 2.

3.3. ^{67}Ga uptake by sex.

Results show that ^{67}Ga uptake is higher in males than females, being 832% in the control group and 1011% in the ID group. In the ID group, males showed greater ^{67}Ga uptake in the following ROIs; (7630%) adrenals, sciatic nerve (6604%), eyeball (995%), heart (361%), small intestine (241%), liver (217%), muscle (139%), blood (122%), peritoneum (117%), gonads (96%), kidney (68%), stomach (53%), and bone marrow (16%) (see Table 1).

3.4. ^{67}Ga uptake by sex / exercise

As mentioned above, exercise increased the ^{67}Ga uptake in ROIs, regardless of the group or sex. Uptake increased 440% in females+EX, 55% in males+EX, 1622% in females-ID+EX and 12% in males-ID+EX when compared to the control group without exercise, which indicates that the impact of exercise is higher in females. The uptake in females ID+EX was 75% higher than females+EX. The differences per sex were higher for the ID group than the control group as shown in table 1.

3.5. Iron binding to hemoglobin

Results showed that ID rats present less Fe bound to hemoglobin than control (Table 2). EX increases Fe-Hb levels in ID group. However, males tend to show higher levels of Fe-Hb than females, with the exception of the control group, although said differences were not statistically significant. Nevertheless, in the ID group a statistical significance was observed, as shown in table 2.

4. Discussion

^{67}Ga is an iron analogue and a partial antagonist of transferrin receptors, and may use the same mechanisms implicated in iron metabolism. It is worth mentioning that ^{67}Ga has not been used as a biosensor of iron biodistribuition or under ID conditions and/or exercise. Cellular metabolism and sex determine the biodistribution of iron; the present study showed that males had higher ^{67}Ga uptake under normal nutrimental conditions, although Fe-Hb levels were slightly lower than in females. This could be because males present major: hematocrit, total hemoglobin, muscle mass [32], TfR-1 expression, liver size, erythropoiesis and testosterone levels; these last two processes inhibit the expression of hepcidin [7,33] promoting iron absorption and mobilization. It is important to mention that a significant proportion of iron in males is used for erythropoiesis, which produces less bioavailability for other tissues [7].

^{67}Ga uptake increase under ID conditions regardless of the sex, this is associated with reports that indicate that low iron levels in plasma decrease hepcidin synthesis [34], increase oxidative stress [35], TfR-1 expression [36] and soluble transferrin receptors (sTfR) [37] promoting the formation of circulating complexes with this transporter protein [38], which may contribute to the increased of ^{67}Ga uptake in blood. This shows that organs capture Fe based on their physiological needs but do not store it, improving the efficiency of systems responsible for iron metabolism.

In ID females, kidney and bone marrow were the ROIs with the highest uptake, demonstrating the importance of iron in erythropoietic and renal functions, possibly at the expense of nerve and reproductive activity due to the fact that ^{67}Ga uptake was greater in gonads and sciatic nerve in females in the control group.

In ID males sciatic nerve and eyeball presented the highest ^{67}Ga uptake, which indicates that iron can be used as an indicator of damage or the specific needs of each ROI, these results coincide with publications that report damage at a nervous [39] or visual [40] level in ID humans. Also, the greater uptake in adrenals could be related to a compensatory mechanism such as extramedullary hematopoiesis; and to alterations in the glucocorticoids and mineralocorticoids synthesis that are associated with depression [41] and blood pressure disorders [42], common problems in ID subjects. Everything mentioned previously could possibly happen at the expense of the reproductive and hematopoietic function, such as what was seen in control males, which presented greater uptake in gonads, kidney and bone marrow.

In fact, with the use of the biosensor it was possible to indirectly identify that the ID subjects, independently of their sex, presented possible gonadal alterations, being ovary and testis the ROIs that show the lowest absorption of ^{67}Ga. This demonstrates that under ID conditions, the small quantity of circulating iron was used by organs with greater metabolic activity, so the ID can be a cause of reproductive problems such as those reported in females [43,44] and males [45,46,47,48,49].

Additionally, exercise increased ^{67}Ga uptake in the ROIs. This is because exercise stimulates a systemic inflammatory state [50], which increases ^{67}Ga absorption in tissues [51]. Inflammation also decreases hepcidin [52] and hemoglobin levels, increasing erythropoiesis [53], and the biodistribution and requirements of iron [54].

Likewise, the results showed that females+EX, regardless of whether or not they were subjected to an ID diet, presented greater ^{67}Ga uptake than males+EX. This means that, when iron mobilization increases in the organism, tissue requirements also increase,

making females more susceptible to suffering the consequences of ID. In ID women who did exercise, it has been demonstrated that hepcidin expression decreases [55] and TfR-1 expression increases [56], this data supports the greater uptake of the biosensor as shown in the present study.

Also, under exercise conditions, ^{67}Ga uptake increases, showing the same biodistribution in both sexes, differing in each group. ID females and males had greater biosensor uptake in sciatic nerve, eyeball and adrenals. This could be linked to the induction of axonal myelination [57] and the increase of the ATPase Na/K concentration [58] caused by exercise, which indicates the importance of iron in the maintenance of nervous and adrenal function in ID subjects, in contrast with the erythropoiesis and the greater hepatic metabolic rate [59] associated with a higher ^{67}Ga uptake in bone marrow and liver in control subjects.

5. Conclusions

Using ^{67}Ga as a biosensor of iron biodistribution, it is possible to identify which organs shows greater or lower needs of iron to perform their functions under normal conditions. Thus, a higher or lower ^{67}Ga uptake in a specific organ may indicate its malfunction or show damage. However, biodistribution varied greatly between females and males, which demonstrates important physiological differences. When subjected to exercise, the biodistribution of the biosensor was similar between females and males of each group (control or ID) although females had a greater ^{67}Ga uptake, phenomena that should be studied in future investigations.

Competing interests

The authors declare that no competing interests exist.

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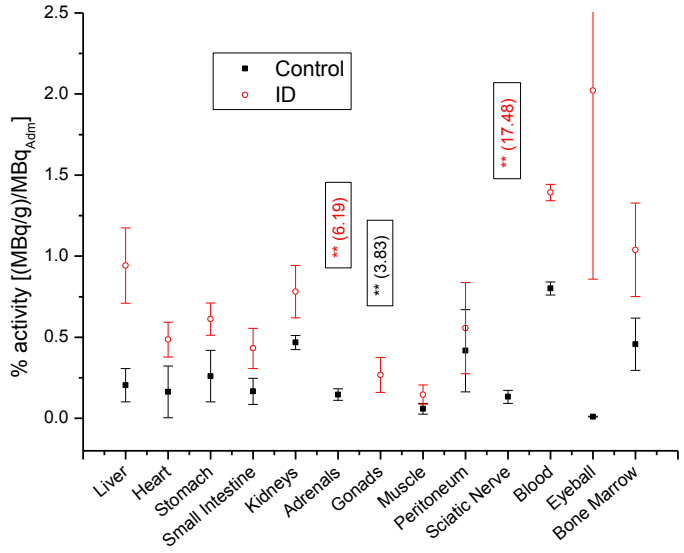

FIGURE 1. ^{67}Ga uptake in the control and deficient groups, n=10 (Error bars are standard deviation). ** *Values out of scale*.

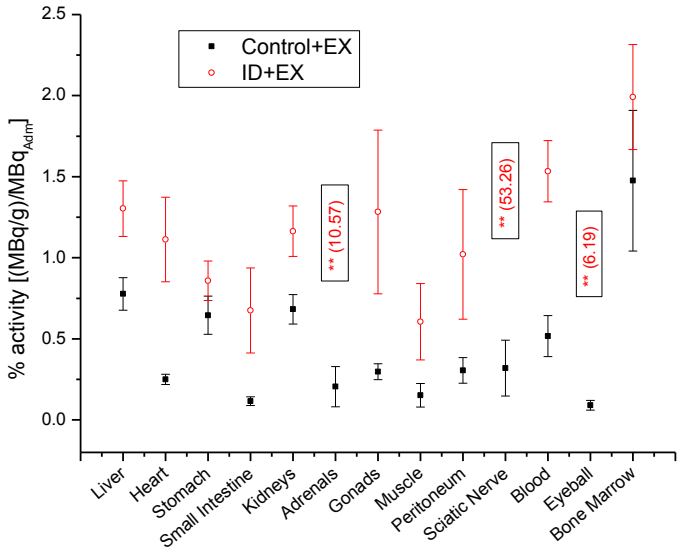

FIGURE 2. ^{67}Ga uptake in the control and deficient groups with EX, n=10. (Error bars are standard deviation). ** *Values out of scale*.

TABLE 1. ^{67}Ga activity in different ROI, 12h post-Injection. Bold text indicates higher ^{67}Ga uptake between females *vs*. males of each group. Asterisks show the group with higher uptake when compared to the control group of the same sex (female ID *vs*. female, female ID+EX *vs*. female+EX; male ID *vs*. male, male ID+EX *vs*. male+EX). The line identifies the ROIs that invert the general uptake performance by group.

Organs	**Males** (MBq/g)/MBq	**Males ID** (MBq/g)/MBq	**Females** (MBq/g)/MBq	**Females ID** (MBq/g)/MBq
Liver	0.3030±0.1355	1.4320±0.3039*	0.1065±0.0539	0.4522±0.1245*
Heart	0.2697±0.2251	0.7978±0.1512*	0.0566±0.0197	0.1732±0.0039*
Stomach	0.4795±0.2241	0.7397±0.1394*	0.0414±0.017	0.4842±0.0149*
Small Intestine	0.1754±0.0719	0.6676±0.1711*	0.1578±0.0891	0.1957±0.0398*
Kidneys	0.8758±0.0560	0.9779±0.2174*	0.0597±0.0265	0.5835±0.0669*
Adrenals	0.2924±0.0096	12.236±1.4681*	0.1682±0.0081*	0.1583±0.008
Gonads	7.6673±2.2515*	0.3543±0.1483	0.2124±0.0424*	0.1804±0.0312
Muscle	0.1047±0.0469	0.2067±0.0836*	0.0146±0.0025	0.0866±0.0011*
Peritoneum	0.6582±0.3538	0.7633±0.2590*	0.1760±0.0575	0.3513±0.3015*
Sciatic Nerve	0.0875±0.0165	34.458±18.318*	0.1777±0.0074	0.514±0.4984*
Blood	0.4633±0.2786	1.9195±0.0639*	0.7011±0.1027	0.8651±0.0321*
Eyeball	0.0214±0.0045	3.7043±1.6445*	0.0196±0.0062	0.3383±0.0161*
Bone Marrow	0.7987±0.2160	1.1174±0.3175*	0.1156±0.0746	0.9607±0.2567*
Organs	**Males+EX**	**Males ID+EX**	**Females+EX**	**Females ID+Ex**
Liver	0.7424±0.1134	1.1308±0.2002*	0.8122±0.0854	1.4760±0.1363*
Heart	0.2481±0.0201	1.0989±0.2896*	0.2524±0.0403	1.1270±0.2282*
Stomach	0.6952±0.1311	0.8079±0.1433*	0.5965±0.1038	0.9092±0.0956*
Small Intestine	0.1185±0.0358	0.4283±0.0337*	0.1134±0.012	0.9220±0.3702*
Kidneys	0.6292±0.0565	1.1402±0.0808*	0.7364±0.1149	1.1871±0.2047*
Adrenals	0.1971±0.0892	8.7535±1.8327*	0.2139±0.1508	12.388±0.9789*
Gonads	0.2016±0.0171	0.5032±0.1464*	0.3938±0.0670	2.0627±0.7274*

Muscle	0.1439±0.0691	0.6078±0.2334*	0.1609±0.0758	0.6030±0.2369*
Peritoneum	0.2972±0.0449	1.0838±0.5557*	0.3140±0.1026	0.9588±0.1075*
Sciatic Nerve	0.4004±0.1474	44.065±8.3354*	0.2393±0.1940	62.454±22.632*
Blood	0.46±0.1223	1.1304±0.2492*	0.5746±0.1290	1.9372±0.0943*
Eyeball	0.1164±0.0342	4.1176±0.1416*	0.0651±0.0249	3.7317±0.4389*
Bone Marrow	1.2818±0.4392	1.7162±0.1557*	1.6698±0.4283	2.2658±0.4312*

TABLE 2. - Iron bound to haemoglobin.

All values are expressed as mean ± SD (standard deviation). Significant results: [a] *vs*. Control (p≤0.01), [b] *vs*. ID (p=0.033), [c] *vs*. Female control (p=0.031).

Fe bound Hb	**By group (mg/kg BW)**	**By Sex (mg/kg BW)**	
		female	**male**
Control	3.68±0.07	3.71±0.11	3.63±0.10
Control+EX	3.54±0.03	3.48±0.03	3.61±0.03
ID	3.28±0.12[a]	3.18±0.23[c]	3.37±0.10
ID+EX	3.65±0.10[b]	3.51±0.11	3.58±0.06