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Original research article

Biodegradable seeds of holmium don't change neurological function after implant in brain of rats

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ARTICLE INFO

Article history:
Received 29 June 2016
Received in revised form
12 February 2017
Accepted 27 March 2017
Available online 11 May 2017

Keywords:
Brachytherapy
Tumor
Seeds
Holmium
Brain
Morphometry

ABSTRACT

Aim: To evaluate the surgical procedure and parenchymal abnormalities related to implantation of ceramic seeds with holmium-165 in rats' brain.
Background: An effective method of cancer treatment is brachytherapy in which radioactive seeds are implanted in the tumor, generating a high local dose of ionizing radiation that can eliminate tumor cells while protecting the surrounding healthy tissue. Biodegradable Ho^{166}-ceramic-seeds have been addressed recently.
Methods and materials: The experiments in this study were approved by the Ethics Committee on Animal Use at the Federal University of Ouro Preto, protocol number 2012/034. Twenty-one adult Fischer rats were divided into Naive Group, Sham Group and Group for seed implants (ISH). Surgical procedures for implantation of biodegradable seeds were done and 30 days after the implant radiographic examination and biopsy of the brain were performed. Neurological assays were also accomplished to exclude any injury resulting from either surgery or implantation of the seeds.
Results: Radiographic examination confirmed the location of the seeds in the brain. Neurological assays showed animals with regular spontaneous activity. The histological analysis showed an increase of inflammatory cells in the brain of the ISH group. Electron microscopy evidenced cytoplasmic organelles to be unchanged. Biochemical analyzes indicate

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http://dx.doi.org/10.1016/j.rpor.2017.03.003

there was neither oxidative stress nor oxidative damage in the ISH brain. CAT activity showed no difference between the groups as well as lipid peroxidation measured by TBARS. *Conclusions:* The analysis of the data pointed out that the performed procedure is safe as no animal showed alterations of the neurological parameters and the seeds did not promote histological architectural changes in the brain tissue.

1. Background

Tumors of the central nervous system are a medical challenge, particularly those of rapid progression, such as glioblastoma multiform, and brain metastasis.[2] Recent scientific advances in diagnosis and disease control have not yet been sufficient to provide a cure for cancer, although remission occurs in many cases.[3] Thus, the search for new treatment modalities is still required.[4–6]

Brachytherapy is an efficient method for cancer treatment,[7] deploying discreet radioactive segments (seeds) in the tumor and generating a high local radiation dose, which is able to eliminate tumor cells while preserving the healthy surrounding tissue. The potential of this therapy justifies the development of new procedures of radioactive implants in tumors.[4,8,9]

Implants made of bioactive materials have been used in medicine and dentistry since the 1990s.[10] The mechanism by which the tissue connects to the implanted material is directly related to the tissue response, the implant interface and the topography of the material.[11] Resorbable implants are designed to gradually degrade with time and be replaced by local tissue, thus reducing the likelihood of side effects on the organism.[12,13]

Bioactive radioactive seeds are a new concept.[3–5,13,14] Ceramic seeds incorporating Sm-152 and Ho-165 isotopes were processed by the sol-gel method.[14] After neutron exposure, the radionuclides Sm-153 or Ho-166 are activated in the ceramic seeds. Those nuclides will decay by emission of high-energy β particles along with γ radiation of 103 keV from Sm-153 and 80 keV from Ho-166, with half-lives of 46.3 h and 26.8 h, respectively.[4,15] Radioactive seeds implanted into tumors of the central nervous system will deposit high doses near the implant, turning in stable isotopes after 8–10 half-lives, i.e. nearly a week, transforming it into a cold material. After cancer cells elimination, the surrounding health tissues will be in contact and interact with such ceramic material during a long-term period. Therefore, short half-life radioactive seeds provide a quick, weeklong brachytherapy followed by a long-term non-radioactive tissue interaction. In this stage, the safety and the toxicity of the cold Ho-165 ceramic seeds in the central nervous system is unknown and may bring some concerns.

2. Aim

Investigations regarding the interaction of cold ceramic seeds with holmium-165 and their effect on the parenchyma of the brain tissue that hold seed implants are not understood. The aim of this study was to investigate the safety of the procedure of the ceramic seed implants with holmium-165 in the central nervous system. Therefore, the surgical procedure and parenchymal abnormalities related to implantation of ceramic seeds with holmium-165 in rats' brain will be investigated.

3. Materials and methods

3.1. Animals

Animal care and experimental procedures were approved by the Ethics Committee on Animal Use (CEUA) at the Federal University of Ouro Preto (UFOP), under protocol number 2012/034, and followed the rules established by the Brazilian Society of Laboratory Animal Science (SBCAL). During all experiments, the animals were kept at room temperature ($21 \pm 2\,^{\circ}C$) and under controlled light cycles in a vivarium at the School of Nutrition (UFOP), with food and drinking water *ad libitum*. Twenty-one adult Fischer rats were divided into Naive Group – 7 Fischer rats that received no surgery, Sham Group – 7 Fischer rats that underwent the surgical procedure without implantation of seeds, and Group with implantation of seeds of Holmium (ISH) – 7 Fischer rats that were subjected to the surgical procedure and the implantation of seeds. 30 days after the animals underwent surgery for implantation of holmium seeds in the brains, radiographic examination was performed. Later, the animals were euthanized by an overdose of anesthetics – sodium pentobarbital, 100 mg kg^{-1}, their intramuscular and brain tissues were collected for analysis.

3.2. Bioactive micro-seeds

Inactive Ho-165 micro-seeds were processed by the sol-gel method following the proposed protocol.[13] The entire process was laboriously accomplished in vacuum, eliminating inherent defects in the seed. After mixing the reagents, the solute-filled molds were prepared to produce micro-seeds of 1.6 mm length and 0.5 mm diameter. Many methods for production of molds were used in an attempt to obtain the smallest seed of acceptable size for the interstitial brachytherapy implant. The vacuum preparation was necessary to avoid formation of bubbles and to allow the fluid to fill the micro holes of the mold. The gelation process, aging, and thermal treatment were accomplished in agreement with methods described in the scientific literature.[10]

3.3. Surgical procedure

After anesthesia and preparation of the animals, the surgical drapes were placed. A median longitudinal incision was made in the animal scalp with about 1.0 cm of length. The opening of plans – skin, subcutaneous tissue, galea aponeurotica and sub-aponeurotic tissue – occurred in the longitudinal axis.

Following the permanent interstitial implantation, double-tipped hypodermic needles were used as guides for placement of the seeds in the brain. The procedure involved the drilling of the frontal bone, following the direction of bregma at 1.35 mm from the sagittal suture and 1.52 mm from the coronal suture. We introduced 3.15 mm of an n° 8 hypodermic puncture needle in the frontal lobe charged with three biodegradable seeds. The incisions were closed with three simple interrupted sutures using nylon 4-0. The animals recovered from surgery in an individual cage with water and food.

3.4. Radiological control

Thirty days after implant, the animals were restrained and anesthetized and X-rays were carried out (60 kV, 70 mA for 0.15 s). The anesthesia was applied in the upper area of the lower limb. The radiographic images were performed in the Department of Nuclear Engineering; Engineering School at the Federal University of Minas Gerais.

3.5. Neurological assay

A modified scale of Park et al.[16] was used to evaluate neurological function following surgery with implantation of seed and no implantation.[17] The animals were evaluated on spontaneous movement, symmetry of limb movements, extension of the front leg and body movement, clamping force in wire cage and proprioception climbing body.[18,19] The bar balance test evaluates motor and vestibular function quantifying the rats' ability to balance on a narrow beam of wood (diameter of 1.5 cm) for 60 s. The parameters are in relation to the support of the legs and their movements over the bar. The Balance Test Score is described as follows: 3 points to 4 well-supported feet, 2 points for two well-supported feet and 1 for the absence of movement on the bar.[20–22]

3.6. Histological staining and morphometric analysis

After fixation, the brain tissue samples were processed according to routine histological techniques and embedded in paraffin blocks. Paraffin blocks were sectioned using a microtome into 4 μm thick sections, placed onto glass slides, and fixed for histological staining. Staining was performed using hematoxylin–eosin and Masson's trichrome stains (Bio-Optica, Milano, Italy).

The whole morphometric analysis was performed at the Multiuser Laboratory of the Research Center for Biological Sciences, at the Federal University of Ouro Preto. To count the number of inflammatory cells in the brain tissue, we randomly obtained 20 images among the histological slides that were prepared from the brain sections. These slides were scanned using the Leica Application software, and analyzed using the Leica Q-Win Plus software (Leica Microsystems, Inc., Buffalo Grove, IL, USA).[23]

3.7. Antioxidant enzyme activity: superoxide dismutase (SOD), and catalase (CAT)

Brain homogenates were used to determine the activity of important enzymes in the tissue's antioxidant defense process. The activity of SOD was determined by measuring the inhibition of autoxidation of adrenaline at 480 nm absorbance, represented by U/mg of protein.[24] CAT activity was measured from the hydrogen peroxide decrease rate at 249 nm absorbance, represented by U/mg of protein.[25] The total protein content of the samples was measured by the method of Lowry.[26]

3.8. Electronic microscopy

The samples were hydrated and emerged in a 1% osmium tetroxide solution for about 2–3 h. After this procedure, the samples were gradually dehydrated until the level of a100% ethanol was reached. Samples were blocked with Tissue-Tek and cut at a cryostat. Slides were prepared for visualization under a microscope.

3.9. Statistical analysis

All data was presented as mean ± standard error of the mean. Statistical analysis was performed using the ANOVA test, unpaired for comparison between the groups. The software used was GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Differences were considered significant at $p < 0.05$.

4. Results

4.1. Biometric analysis

The animals were weighed prior to their surgical procedure and 30 days after it, on the day of euthanasia. There was no difference between the three groups after surgery with respect to weight of the animals.

4.2. Neurological tests

Neurological tests were performed in order to exclude any injury resulting from the surgical procedure or seed implantation.[1] The tests occurred in three distinct stages: 24 h after surgery, 15 days after surgery, and 30 days after surgery. There were no neurological changes in the parameters evaluated between animals of both groups (Table 1).

4.3. Radiological examination

Radiographic examination was carried out on animals in the ISH group with the following specifications: 60 kV, 70 mA for 0.15 s. In the radiographic examination, it was possible to confirm the location of the seeds through the visualization of a radiopaque area in the upper region of the skull, at the frontal lobe (Fig. 1).

Table 1 – Evaluation of Fischer rats neurological behavior (adapted Garcia et al.).[17]

Function		Score	ISH	SHAM	*Naïve*
Spontaneous activity	Normal	3	X • #	X • #	X • #
	Little affected	2			
	Greatly affected	1			
	No movements	0			
Symmetry in motion	Symmetric	3	X • #	X • #	X • #
	Asymmetric	2			
	Hemiplegic	1			
Movement when the animal is held by the tail	Paws symmetric	3	X • #	X • #	X • #
	Slight asymmetry	2			
	Marked asymmetry	1			
	Forelimb did not move	0			
Climbing for observing the clamping force	Rose/grabbed easily	3	X • #	X • #	X • #
	One side hurt	2			
	Failed to go up or tend to circulate	1			
Proprioception body	Same on 2 sides	3	X • #	X • #	X • #
	Slow reaction to stimuli of 1 side	2			
	No response 1 side	1			
Balance (wooden bar 1.5 cm in width)	4 legs well supported	3	• #	X • #	X • #
	2 legs well supported	2	X		
	No movements	1			

4.4. *Histological analysis*

Through quantitation of inflammatory cells in the brain parenchyma, we can observe differences ($p < 0.0001$) regarding the number of inflammatory cells in the brain of groups SHAM and Naïve when compared to group ISH (Naive: 4 ± 1; SHAM: 5 ± 1; ISH: 20 ± 2; cells per microscopic field, $p < 0.0001$; Fig. 2). The tissue's architecture remained preserved and there was no significant collagen deposition. As in electron microscopy, images evidenced cytoplasmic organelles to be unchanged (Fig. 3).

4.5. *Biochemical analysis*

The enzymes with antioxidant function showed no change in their activity, indicating that there was no oxidative stress in the parenchyma of the ISH animals. The activity of catalase

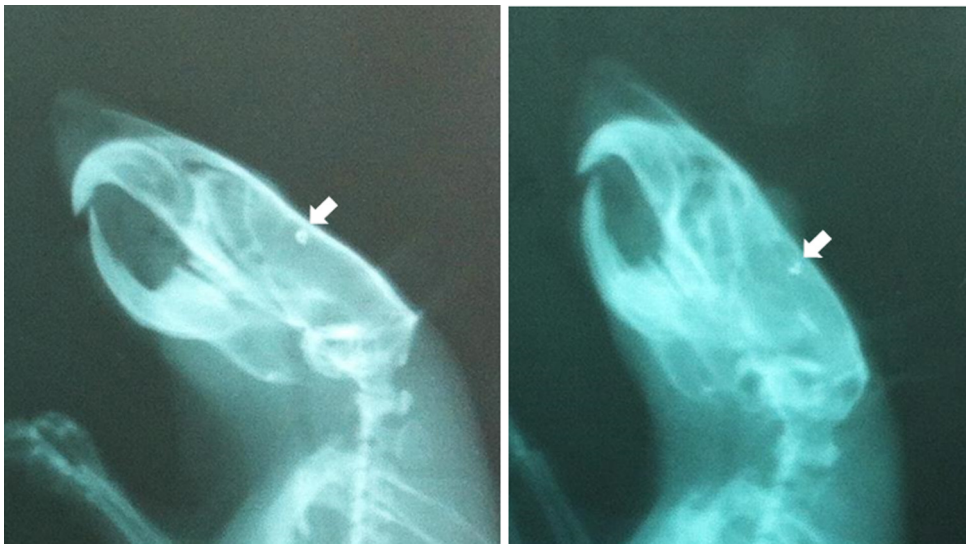

Fig. 1 – Radiograph of the skull of the animals of the group with the implantation of biodegradable Holmium seeds (ISH). It was possible to confirm the location of the seeds through the visualization of a radiopaque area in the upper region of the skull, at the frontal lobe. Holmium radiopaque seeds in animals that received it (arrow).

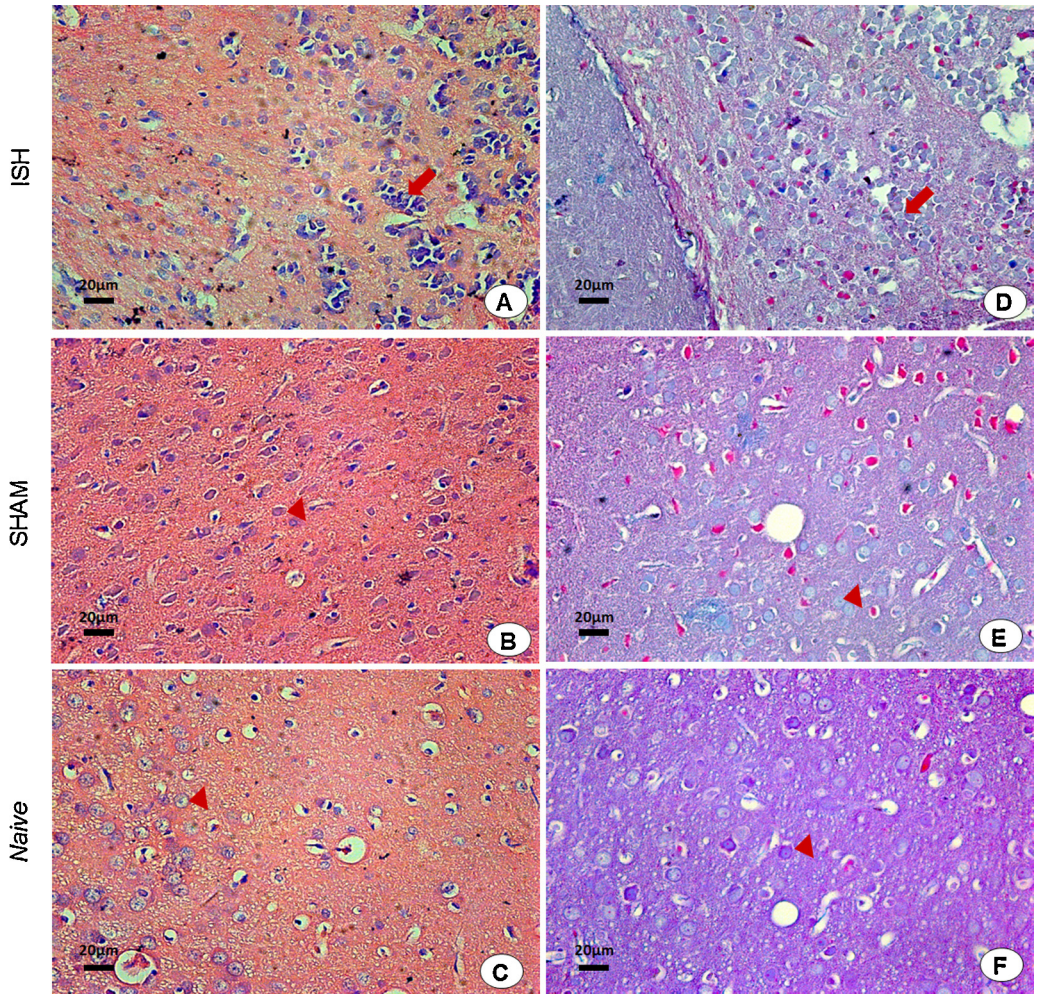

Fig. 2 – Photomicrographs of rats' brains. A and D – Arrows indicate the presence of inflammatory cells in a group with implantation of biodegradable Holmium seed (ISH) but the absence of collagen fibers (H & E and Masson's Trichrome, respectively). B and E – Group that underwent surgical procedures without implantation of seeds (SHAM): arrowheads indicate neuron cell bodies, demonstrating the preservation of the brain parenchyma (H & E and Masson's Trichrome, respectively). C and F – Animals with absence of surgical procedure (Naive): brain parenchyma preserved. 440×; Arrowhead: neuron cell bodies; Arrow: inflammatory cells, BV: Blood Vessels; Bar: 20 μm.

was unaltered in all the groups ($p = 0.0531$) and lipid peroxidation measured by TBARS remained similar ($p = 0.2642$) (Fig. 4).

5. Discussion

The tumor approach through brachytherapy creates hope for unresectable cases, either primary brain tumors or metastases from other sites.[27,28] Iodine-125 brain brachytherapy has been recently reviewed.[30] The method provides encouraging survival rates with relatively low complication rates and good quality of life.

Brachytherapy with metallic iodine-125 seeds has already been performed in the prostate and in brain organs. Iodine-125 has a half-life of 59 days, providing 36-keV gamma emitted at a low dose-rate. The imparted total dose of iodine-125 seeds is achieved only after a year of exposure. The 1.6 mm × 0.5 mm Ho-165 seeds have 1/8 of the volume of the 4.5 mm × 0.8 mm iodine-125 seed and provide 1.85 MeV beta-emitting with 8.7-mm maximum range, depositing its energy with 26.8 h half-life, which means a high dose-rate and total imparted dose deposited in a week. Large beta-particle energy implies lower numbers and total activity of Ho-166 seeds in comparison to iodine-125 seeds to perform similar dosimetry. Implants with Ho-166 seeds will provide a similar clinical response; however, with a low number of seeds in the implant. Also, dose homogeneity in the target-volume shall be improved, providing better dose distribution. It shall be due to the beta dose profile from seed surface, which do not obey an exponential function such as I-125 does. In addition, dose in surrounding tissues a few centimeters away from implants will be negligible since beta particle has a limited range.

Large diameter and long needles used on the I-125 brachytherapy are quite aggressive to the organs. Implants with iodine-125 seeds are performed with the help of an acrylic template of a set of 2-mm holes, each separated by 10 mm, in

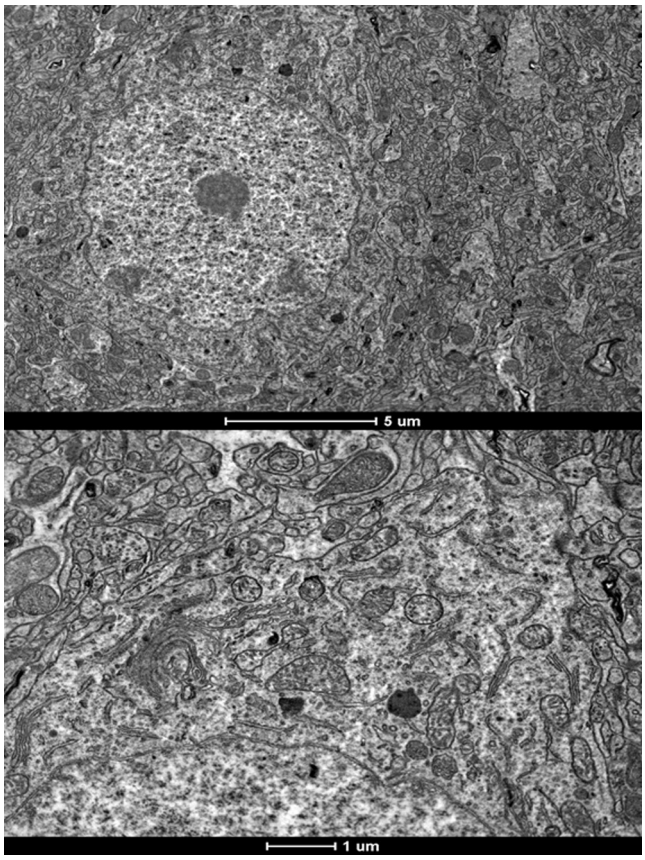

Fig. 3 – Electron microscopy of neurons of animals with implantation of biodegradable seed Holmium (ISH). Neuronal bodies with cytoplasmic structures preserved. Mitochondria with normal crests, vesicles, Golgi Complex and endoplasmic reticulum preserved.

which a set of 1.2 mm-thick needles are inserted. The similar protocol can be used to implant Ho-166 seeds, however, with a template with 0.8 mm-diameter holes, each separated by 10 mm. Thin needles can be used to placing a sequence of seeds spaced by 10 mm. A low number of needles is expected for Ho-166 implants. Also, stereotactic brachytherapy can be used in both types of implants.

Synthesis of holmium seeds has already been investigated exhaustively.[8,31–33] Elementary weight concentration of the seeds was previously analyzed. The cylindrical seeds identified also as [Si:Ca:Ho] were synthesized by the sol–gel method. The chemical concentration values for Ho in [Si:Ca:Ho] seed was found to be 23.1 ± 0.7% by weight by UV–vis spectrophotometry[3,33]; and, 21.5 ± 0.5% by INAA and 23 ± 3% by ICP-AES.[8,31,32] On average, the Ho concentration was 22.5 ± 0.7%.

The tissue-ceramic biocompatibility is a requirement to have the bioactive material adherent to host tissue, guarantee stable position and lower inflammatory response. Biodegradability is a long-term response to the stable, non-radioactive bioactive ceramic material. Biocompatibility and biodegradability features may represent a better quality of life for patients with brain implants. Survival depends on the covering of the target-volume by a prescribed dose and its spatial distribution cannot miss infiltrations. A set of Ho-166 seeds well-placed and distributed on the target volume may improve survival.

Radiological response of ceramic implants has already been investigated.[4,5,35] Conventional X-ray, mammography, ultrasonography and computer tomography have been shown to be suitable image techniques to depict ceramic seeds on organ implants. In addition, ceramic seeds do not generate artifacts as star profiles well known in the case of metallic seeds in CT images.

In the present study, the implants were not radioactive. The stage of the research is to investigate the safety of the implants in long-term period. The implants are performed and imparted dose is achieved in 10 half-life of the radionuclide, which is performed in a short-period of time. After that, the cold seeds are kept in place interacting with the healthy brain in a long-term period. The study addressed this period in order to guarantee the safety of the procedure in a long-term period.

Recently, brachytherapy planning of Ho-166 was compared to HDR Ir-192 brachytherapy in breast cancer.[34] It was shown that breast Ho^{166}-implants of 37–111 MBq are suitable due to the high-dose rate near 6–10 mm from seed surface, similar to Ir^{192}-brachytherapy of 259 GBq (3 fractions, 6 min). Doses in standard points were similar after a week of exposure. The spatial dose distribution of Ho-166 was confined, better than Ir-192.[34]

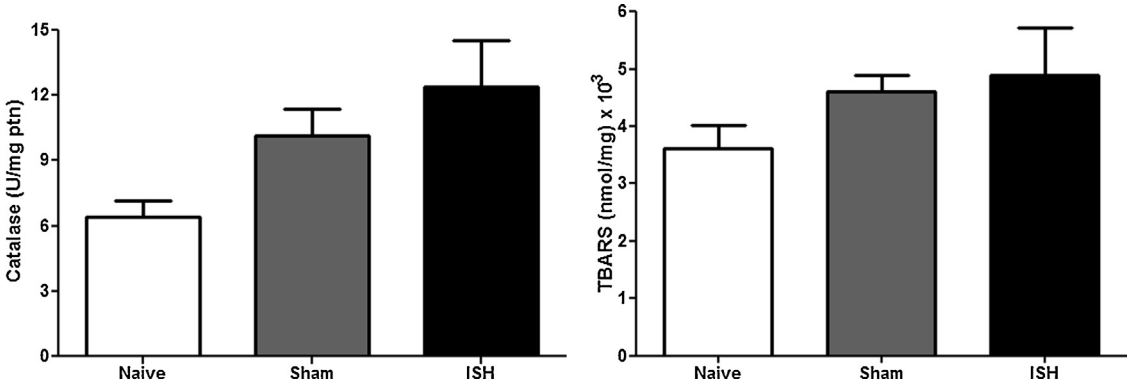

Fig. 4 – Measurement of catalase activity and thiobarbituric acid reactive substances activity. The activity of catalase did not change between the groups ($p = 0.0531$) and lipid peroxidation measured by TBARS remained similar ($p = 0.2642$).

The maximum range of β particles emitted by Ho-166 was evaluated assuming a maximum energy of 1854.7 keV.[3] The mass extrapolated range R_{ex} in $g\,cm^{-2}$ was $0.867\,g\,cm^{-2}$ in water. In addition, the irradiated volume is approximately a sphere with a radius of 8.7 mm. Implants in the liver of rabbits made of bioglasses of [Si,Ca,Sm] had been prepared previously.[13] The ultrasound was able to identify the seeds in the liver, due to its high ecogenicity. CT images of the thoracic-abdominal area of the rabbit were taken with the seeds. The absorption of the seeds was investigated in the organ. The seeds of [Si,Ca,Sm] showed to be absorbed in the liver after radiological exploration seven months later. The biodegradability was certified by X-ray.

Holmium-166 radionuclide has already been applied to brachytherapy. In a study by Bult et al.,[29] liver microbrachytherapy with holmium-166 microspheres improved survival, weight and fur of animals with unresectable liver tumors. Due to the large number of deaths, Ho-166 brachytherapy could also bring benefits to the treatment of tumors in the central nervous system. Indeed, Ho-166 seeds seem to be a good choice.

The present paper addressed the safety and toxicity of the Ho-165 seed implants in rat's brain, in a long-term period (30 days) that always follows after brachytherapy. Our findings indicate that the procedure is safe as the animals showed no neurological changes regarding the parameters evaluated and the seeds did not alter the brain architecture or the neuronal enzyme activity.

6. Conclusions

Our results are preliminary but suggest that there was no biometric change after implantation of radiation seeds in the animal's brain. Neurological tests showed that the procedure performed is safe as no animal showed alteration on the evaluated parameters. There was an increase in the number of inflammatory cells but there were no collagen deposition in the brain parenchyma. Biochemical testing indicates that there was neither oxidative stress nor oxidative damage in the brain of animals that had undergone implantation of biodegradable seeds with holmium-165. Under electronic microscopy, it was possible to identify the preservation, at cytoplasmic level, of neuronal organelles. However, further analyzes are still needed to evaluate the viability of seed use.

Conflict of interest

None declared.

Financial disclosure

None declared.

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