Joint Institute for Nuclear Research.

Laboratory of Radiation Biology.

FINAL REPORT ON THE START PROGRAMME

" The study of the side effects of whole-brain irradiation on functions, behavior, and physiology of Sprague Dawley rats: A comparative study."

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Abstract

Radiotherapy is useful for specific types of cancers as a type of treatment strategy. On the other hand, it has side effects on the brain. Patients who get radiotherapy for brain cancer can suffer from fatigue, hair loss, memory or concentration problems, nausea, vomiting, skin changes and blurry vision. To be more specific, during this internship, we studied the efficacy of diverse types of radiation on functions, behavior and physiology of Sprague-Dawley rats. Through proceeding the acute changes that happened to the rat brains, their behavioral functions, and histopathological changes, we figured out that the rats irradiated with 8Gy suffered from anxiety ($p=0.031$) and this was observed through frequent entries of the central zone. In the histopathological analysis, it was observed that the %area of myelin distribution in the 4Gy irradiated rats was lesser than the control group which indicates the damaging effect of irradiation on the myelin sheath of this group.

Introduction

Radiation is the transfer of energy from one location to another in the form of waves or particles. We are exposed to radiation throughout our daily lives. Some of the most common sources of radiation are the sun, microwave ovens in our kitchens, and the radio in our cars. Most of this radiation poses minor damage to human health. But some do. Radiation has a lesser risk at lower doses but can increase at greater exposures. Depending on the type of radiation, various precautions must be taken to safeguard our bodies and the environment from its effects while yet allowing us to benefit from its numerous applications, for example it is used in medical procedures such as many cancer treatments which are called radiotherapy. Recent investigations have demonstrated that the brain is certainly vulnerable to irradiation. Cranial radiation therapy has a wide range of effects on brain processes, resulting in cognitive decline, memory problems, fatigue, and brain tumors in the exposed individuals. The extent and severity of radiation's effects on the brain depend upon the radiation dose [1, 2].

CNS dysfunction in patients after irradiation is multifactorial, influenced by individual factors [3, 4] including age, medical comorbidities, psychological and genetic predispositions, characteristics of any underlying malignancy, as well as any additional injuries caused by other treatment modalities such as surgery and chemotherapy. From a radiobiological perspective, radiation-induced brain injury is described in three phases: acute (within days to weeks after irradiation), early-delayed (within 1–6 months post irradiation) and late (> 6 months post irradiation) [5]. Using radiotherapy causes functional toxicities that have correlation to observe changes in the entire brain, including gray matter, white matter, ventricles, and combinations among them [5, 6]. Hallmarks of normal tissue toxicity include vascular injury [7]. Radiation primarily causes coagulation necrosis of the white matter tracts and cerebral vasculature by axonal demyelination and damage to vascular endothelial cells [8]. Leukoencephalopathy occurs from the overproduction of myelin in oligodendrocytes and occurs as a late toxicity. Demyelination can also occur in spinal cord and nerve roots. Neurodegeneration may occur directly from radiation-induced stress as well as a by-product of detrimental effects on the supporting astrocytes and supporting astrocytes may undergo reactive gliosis. However, the most

severe form of injury is radio-necrosis, producing a brisk neuroinflammatory reaction Neuroinflammation is a prominent feature of many CNS diseases including stroke, Alzheimer's disease, Parkinson's disease, and mild cognitive impairment, and has also been hypothesized to contribute to radiation-induced cognitive losses [9, 10]. During my internship, we delved into investigating the efficacy of radiation on Central Nervous System (CNS) especially brain functions and behavioral tests and demyelination in histological samples going through distinct types of radiation i.e., x-rays radiation for SARRP and proton radiation for neuroprotection experiment.

Project goal

During the internship, our objective was to investigate the efficacy of irradiation on rat brain and pituitary gland using several techniques of analysis and interpretation.

Methodology

1- Animals and study design.

A- SARRP Experiment.

Sprague Dawley male rats nine -ten weeks old (for EEG experiment) on time of irradiation, were used during this study. X-ray irradiation for SARRP experiment was used. The Whole-brain irradiation (SARRP) was performed with isoflurane anesthesia. Cranio-caudal direction in plastic stainers showed in **Fig. 1 (a)**. The study was divided into three groups: **Control group**: contained four Sprague Dawley rats. **4Gy irradiated** : contained one rat. **8Gy irradiated**: contained four rats.

B- Neuroprotection Experiment

For Neuroprotection group, 4Gy proton irradiation in the Phasotron JINR in the proximal part of Bragg curve) **Fig.1. (b)** and a specific drug for neuroprotection was used.

In this study, we used Sprague Dawley male rats 33 weeks old divided into four groups:

Group 1:(Proton+ drug): eight rats. **Group 2**: (Proton+ Saline): eight rats.

Group 3: (Control+ drug): eight rats. **Group 4**: (Control+ Saline): eight rats.

2- Radiation Procedures.

Phasotron of JINR was used to irradiate all test group rats included in the experiments.

(a) (b)

Fig.1. (a) Irradiation of Sprague Dawley rats using Cranio-caudal direction in plastic stainers for SARRP experiment. **(b)** The chart shows the proximal part of Bragg curve used to irradiate the rats of neuroprotection experiment.

2- ***Brain Functions and behavioral tests.***

- ***Open Field Test (OFT)***

The open field test is used to analyze locomotion, anxiety, and stereotypical behaviors such as grooming, rearing, freezing, central zone entries, holes dipping, urination, and defecation in rodents showed in **Fig.3**. This test uses a camera to assess the movement of the test animal [11]. It was observed that grooming is when the animal tries to clean its body and shows how the animal is relaxing. Rearing is when the animal stands on its hind feet and raise its frontal feet or uses the wall to stand up. The hole dipping on the ground of the used arena of OFT means exploration. Central zone entry means lower anxiety showed in **Fig.2.(a)**. Defecation and urination mean huge anxiety and stress. Freezing that is when the rat does not move indicates fear. We used the Open field circle shaped test arena with a fence surrounds it shown in **Fig.2. (b)**. The open field test was performed for SARRP and Neuroprotection experiments one day after irradiation.

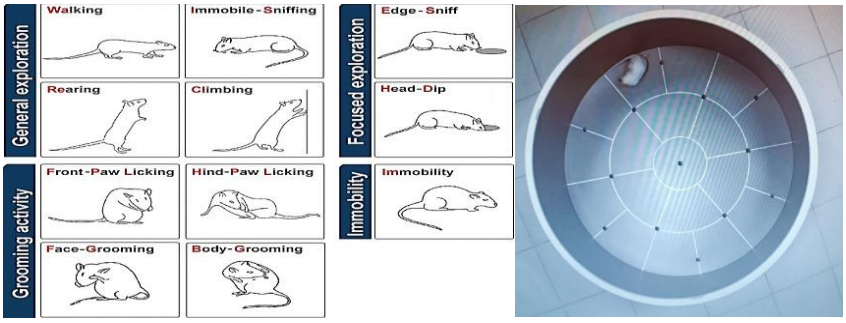


Fig.2. (a) Animal actions during Open Field Test **(b)** The circular arena used for Open Field Test.

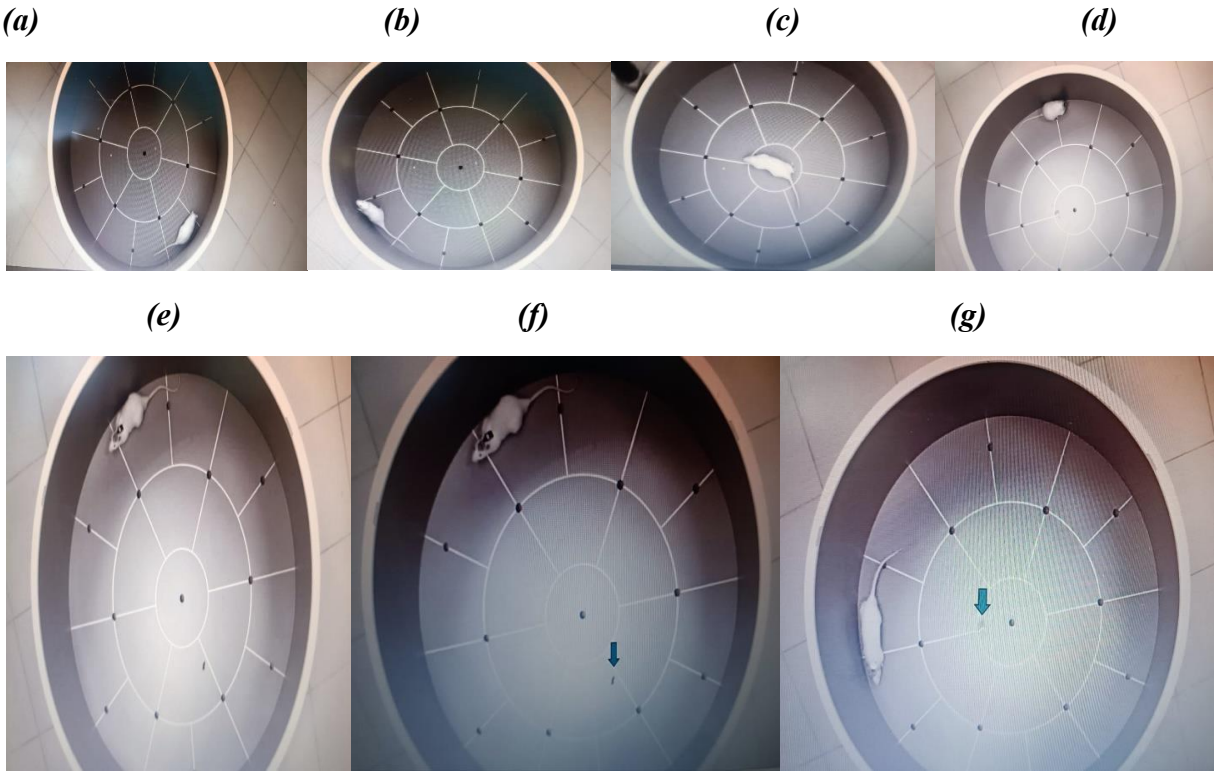


Fig.3. a)Holes ***b)***Rearing ***c)***Central zone entry ***d)****Grooming* ***e)****Freezing* ***f)*** *Defecation* ***g)*** *Urination.*

3- <u>Histopathology procedures.</u>

At the department of astrobiology and physiology, we worked on rat histopathological samples delving into its practical steps as followed:

a- Sacrifice/ decapitation

At the end of the experiments and after 14 days of irradiation, decapitation was performed.

Organs were separated and histopathological procedures were performed starting from perfusion, processing, embedding, and trimming (sectioning) till examining it under the microscope.

b- Perfusion of fixation.

The first step of histological investigation was transcardial perfusion fixation.

- ***<u>Preparation of facility and instruments.</u>***

A perfusion pump was set up in a fume hood a pipe system with bi- or tri-pass valve and needle (blunt needle such as feeding needle), a tray to collect biohazard fixative shown in **Fig.4.a.** and the instruments needed shown in **Fig.4.b.**

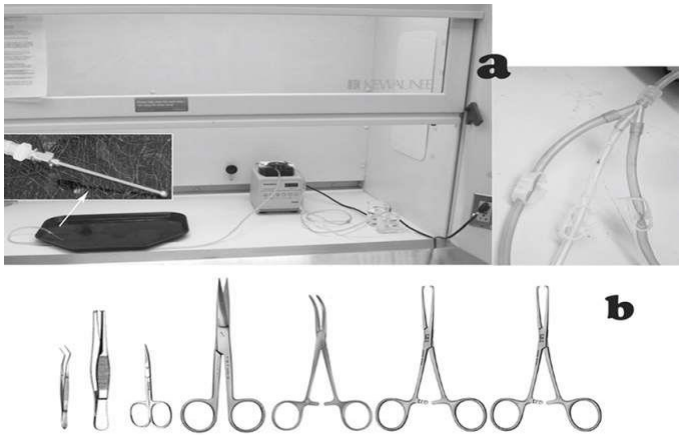


Fig.4. Facility and instruments for transcardial perfusion.

- **<u>Rinse the solution and fixative.</u>**

- Saline (0.9% Nacl)
- Phosphate buffer (PB, PH 7.4) either 0.2M.
- Paraformaldehyde (PFA; powder) or formalin.
- Fixative for EM tissue preparation:
  (2% PFA & 1% glutaraldehyde, and mostly 0.075- 0.2 glutaraldehyde is applied with 4% PFA for immunohistochemistry.

- ***<u>Followed transcardial perfusion protocol.</u>***

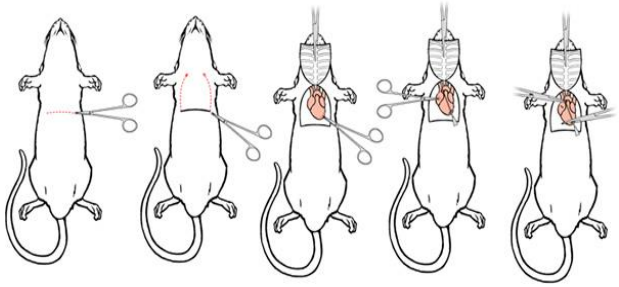


Fig.5. Perfusion surgery steps.

1- Anesthesia of the animal using the available anesthetizer in the animal protocol and for transcardial perfusion, deep anesthesia by double dose is required. Moreover, we must proceed the steps of perfusion after we see the signs of deep anesthesia which include deep irregular breaths, cyanosis (blue color) on footpads, ears, and tail. In this situation, the blood vessels are completely relaxed.

A side note: Excellent anesthesia and perfusion does not require an anticoagulant and Vaso-dilatant, but some researchers administer heparin or sodium nitrile (SN) or both to avoid blood coagulation and reflexive vessel contraction.

2- We started a rapid surgery to open the chest and ascending aorta shown in **Fig.5**. [12].

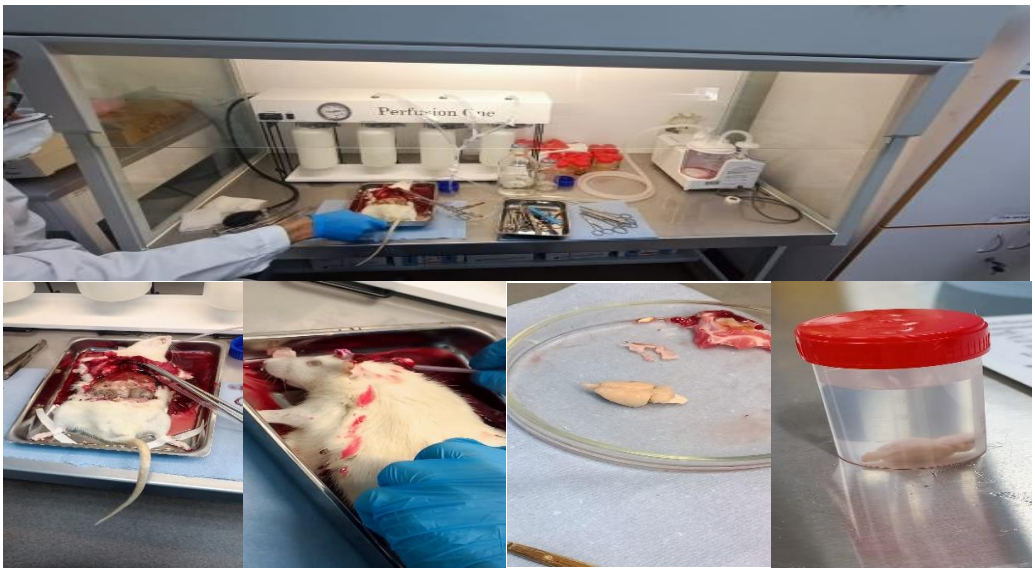
3- We started to pump saline at very slow rate (1-2mL/min) and inserted feeding needle into left ventricle until the tip is in the aorta. Slow pumping made the right auricle plump and easy to see. We cut the auricle and increased the speed on the pump to the fasted rate (e.g., 40mL/min), The saline pump was closed as soon as the blood flowing out of the auricle looked clear and opened the fixative pipe in the meantime.

A side note: We must make sure that there are no air bubbles at the tip of the feeding needle prior to pushing it into the heart. Also, we should examine all the pipes to make sure no air bubbles are inside; if any, we must try to get rid of them before perfusion. We must pay attention to this step because air bubbles can inter into small blood vessel to block flow of perfusion and if this happened in the brain, the brain tissue would not be evenly fixed.

4- We perfused animal with fixative at a fast rate (about 40mL/min). After approximately 5 min of fast perfusion, we turned pump back to 15-20 min, and then reduced flow rate again to about 2mL/min for an additional 30-35 min or until fixative runs up **Fig.6. (a)&(b).**
5- After finishing perfusion steps, we can observe the white eye of the animal as a proof of good perfusion **Fig.6.(c)**. We extracted the brain **Fig.6.(d)** and immersed it in formalin buffered 10% for 24 hours as shown in **Fig.6.(e).**

A side note: A sign of good perfusion is that animal raises up the front limbs and stretch out and rear limbs shortly after fast perfusion of fixative. The saline to be prepared should be 5-10 time of blood volume averagely (7.5 times). The brain should be placed in increasingly gradient sucrose after it is extracted [13].

(a)



(b) **(c)** **(d)** **(e)**

Fig.6. (a)&(b) During perfusion process **(c)** White eye of the rat is a proof of good perfusion **(d)** extracted brain. **(e)** Immersed brain in formalin buffered 10% for 24 hours.

c- Processing and Embedding Protocols

Embedding is the second step of histopathological investigation. It is the process of placing the tissue in molten paraffin before sectioning [14]. Embedding must be performed perfectly to prevent the tissue from damaging during sectioning. After transcardial perfusion, processing and embedding of rat brain tissue was performed (three parts of the brain-2 left sagittal parts and cerebellum). The followed (RITA) protocol for embedding is shown in **table.1.**

Tap water	˜1h	Room temperature
Ethanol 50%	1h	Room temperature
Ethanol 70%	1h	Room temperature
Ethanol 95%	1h	Room temperature
Ethanol 100%	2h	Room temperature
Ethanol 100%	2h	Room temperature
Ethanol 100%	2h	Room temperature
Xylene	1h	Room temperature
Xylene	1h	Room temperature
Paraffin	1h	60 deg.
Paraffin	1h	60 deg.
Paraffin	˜1h (overnight)	60 deg.

Table.1. protocol of processing and embedding**.**

d- Trimming/Sectioning

- The third step is sectioning.
- Sectioning is a crucial step during histopathological investigations. It refers to cutting the sample tissue into thin layers or slices to prepare a slide to be examined under microscope .
- To get optimum sectioning results, we learnt two tips:
  - First, we can use our exhalation air through our mouth to make sectioning easy.
  - Additionally, handling the tissue and sample cutting should be gentle. Do not rush!

- When we started sectioning, we wrote down (date, block number, Animal type/ tissue, end point of trimming “Start of Cutting” , trimming depth, number of slices on the slide and thickness of the sample). For sectioning, we used the microtome to make five-micrometer samples as shown in **Fig.7**. **(b)**. We hold the already cut specimen with forceps and let it float in a water bath adjusted to about 45°C **Fig.7. (c)**. We bring a glass slide to the water bath to pick up the specimen on it, and let it dry at nearly 45°C **Fig.7. (d)**. After this, it is left for 24h at room temperature for a proper fixation. In histopathology, each step must be done properly, so as not to affect the other steps and to prepare spectacular slides to be examined without artifacts.

(a) *(b)* *(c)* *(d)*

***Fig.*7. (a)** During proceeding the process of microtomy. **(b)** Microtome. **(c)** Floating samples in the water bath. **(d)** Drying the slides containing the samples for good fixation.

e- Staining

Luxol Fast Blue- Modified Kluver's- Myelin Sheath Staining Protocol.

- Myelin is a critical component of the nervous system white matter. The myelin sheath surrounds axons and thereby provides the necessary insulation for the efficient transmission of neural electrical signals across brain regions [15]. Myelin can be lost in a variety of conditions or disease states. For example, multiple sclerosis (MS) is a common neurological condition resulting in a dysfunctional immune system-mediated loss of myelin, which causes symptoms such as muscle weakness, paresthesias, and fatigue. Traumatic brain injury, such as a concussion, can also lead to demyelination (Inflammation and white matter degeneration persist for years after a single traumatic brain injury).
- Luxol Fast Blue (LFB) is a dye that stains myelin in formalin fixed tissue . LFB provides information on the presence of myelin in terms of its spatial distribution but does not allow quantification. Using a LFB stain was useful here to assess the severity of myelin loss [16].
- After cutting the paraffin sections 5µ, we allow it to dry at 37°c over night.
  1- We deparaffinize the samples using **xylene** thrice each for 6 minutes.
  2- Hydrate them with **95% alcohol** (freshly prepared) for about 5-6 minutes.
  3- We transfer the samples to **Luxol fast blue solution** (LFB) for about 2 hours then we start to check the results and if it needs more time, we leave it for extra time.
  4- After LFB staining, rinse the samples in distilled water.
  5- Transfer the slides to Lithium carbonate solution for 5 seconds.
  6- After this step we should transfer the slides to 70% alcohol, two changes, 10 seconds each.
  7- Immerse the samples in Eosin for counter staining for one minute.
  8- Wash using distilled water.
  9- Repeat the steps from 5 to 7 until there is a sharp contrast between the blue of the white matter and the colorless gray matter.
  10- Rinse in distilled water.

11- Finally, dehydrate through 95% and 100% alcohol, clear in xylene, and coverslip the slides.

Fig.8. *We 'START students' while we were staining the samples.*

Statistics

In SARRP experiment, (Autopsy of brain and Pituitary gland and body weight), Neuroprotection experiment and to analyze the whole data for the used techniques, we used Jamovi 2.5.6. To interpret our results for Open Field Test (OFT),we used Independent T-test (normality test) and according to the results we used Student's Test, Mann-Whitney U Test and One-Way ANOVA Kruskal-Wallis.

Results and discussion

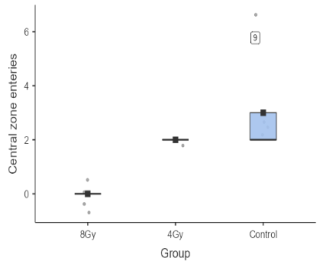
Experiment no.1: SARRP.

When we used (One-way ANOVA) (Non-parametric) Kruskal Wallis statistical analysis, we figured out that ($p = 0.027$) for central zone entries **Fig.9.(a).**which was significant and indicated lower anxiety. It was also observed that ($p = 0.018$) for defecation **Fig.9.(f).**which is also significant and indicated the severe anxiety and stress. When we compared 8Gy irradiated rats with control ($p=0.031$) which is significant and shows that when rats are irradiated with high dose (8Gy), they rats tried to express their stress and anxiety through frequent entries of the central zone entries. For grooming, **Fig.9.(g).**when we compared the 8Gy group with the control one, it was observed that it was less in in 8Gy irradiated rats and as previously mentioned grooming indicates relaxing, that's why 8Gy irradiated rats showed the efficacy of radiation. Freezing indicates fear and when we compared 8Gy group with the control, we found that 8Gy irradiated rats had freezing times more than the control group and the damage of irradiation was obvious shown in **Fig9.(h).**

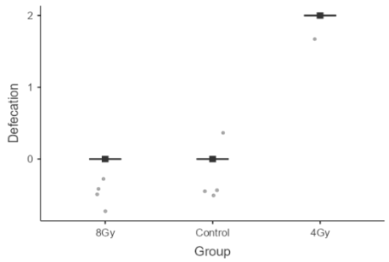
Plots for SARRP Experiment.

Figure.8.

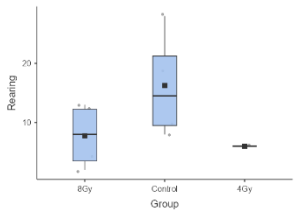
a) Central zone entries of SARRP.



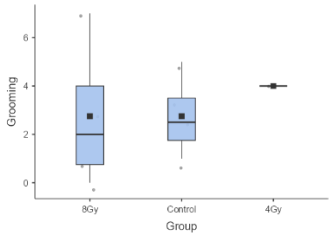
f) Defecation of SARRP.



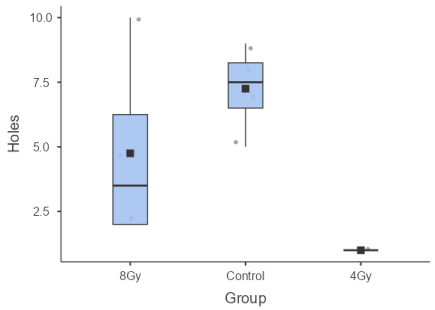
b) Rearing of SARRP.



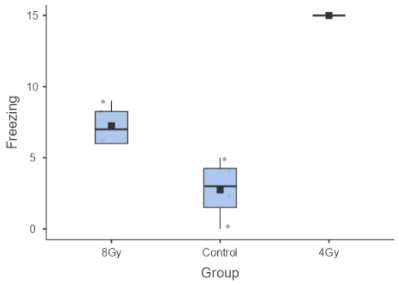
g) Grooming of SARRP.



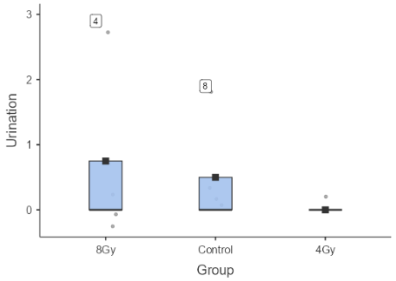
c) Holes of SARRP.



h) Freezing of SARRP.



d) Urination of SARRP.

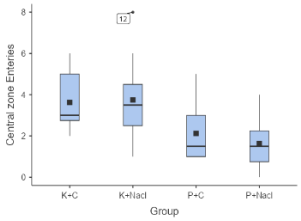


Experiment no. 2 Neuroprotection.

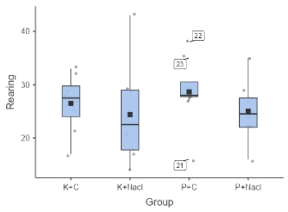
In Neuroprotection experiment, when we used One-way ANOVA (Non- parametric) and due to non-homogeneity, we found that (p= 0.049) which is borderline for the variable (central zone entries. And for the other variables such as rearing, grooming, holes, freezing, defecation, and urination there was no signification between the groups due to the little interpreted changes.

Plots for groups of neuroprotection experiment.

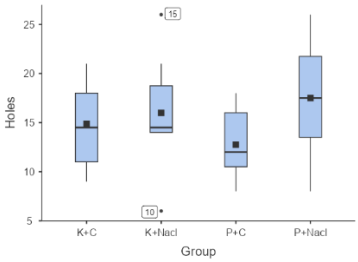
Central zone Entries



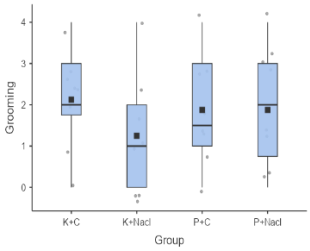
Rearing



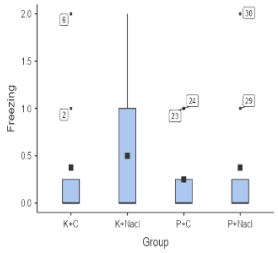
Holes



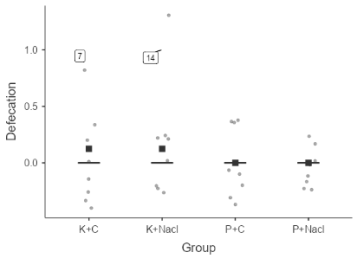
Grooming



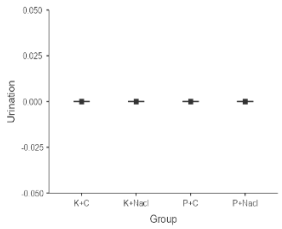
Freezing



Defecation



Urination



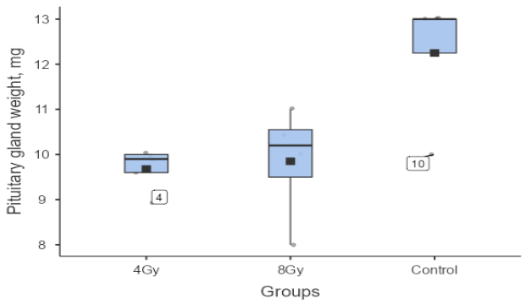
Experiment no.3: Autopsy.

We used the statistical analysis One-way ANOVA (Non-parametric) to determine the changes happened to the body weight, brain weight, and pituitary gland after irradiation. When we compared the 4Gy-irradiated rats to the control group the (p=0.058).

It was observed that the body weight was less in the 8Gy compared to control group shown in **Fig.10.(b).**

Plots of Autopsy Experiment.

a) **Pituitary gland weight, mg**



b) Body Weight, mg. **c) Brain Weight, mg.**

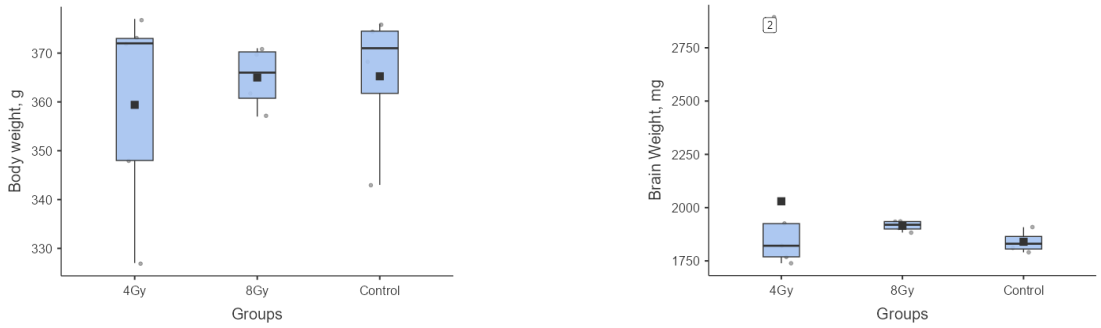


Fig.10. a) This plot shows the difference between pituitary gland weight between 4, 8Gy and control groups of SARRP experiment. **b)** shows the difference between body weight between 4, 8Gy and control groups of SARRP experiment. **c)** shows the difference between brain weight between 4, 8Gy and control groups of SARRP experiment.

Noldus EthoVision XT tracking software for Open field test of SARRP Experiment:

EthoVision XT 13 was for Open Field Test (OFT) tracking and analyzing the results. In **Fig.11.** we can see the movement of the rats. We observe the yellow color that expresses the areas at which the rat stopped moving during OFT . **Fig.11.** shows the heatmaps of the 4Gy, 8Gy irradiated by X-rays and control groups, respectively. The frequent stops or immobility of the rats (the yellow color) indicates the efficacy of radiation on the brain which may express depression of the irradiated rats. On the other hand, the quick movement of the rats can show the anxiety and fear of rats because of irradiation.

(a) 4Gy **(b)** 8 Gy **(c)** Control

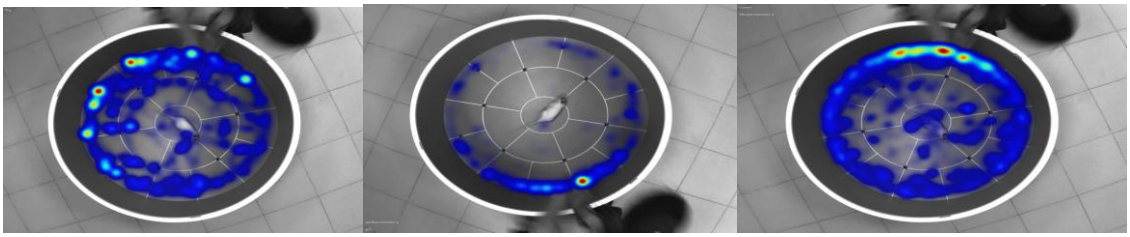


Fig.11. EthoVision XT Heatmaps**: (a)** 4Gy**, (b)** 8Gy**, (c)** Control group

Distance and velocity moved by the rats in SARRP:

- We used student's t test for Independent Samples T-Test to compare between 8Gy and control groups of SARRP experiment and it was observed that distance (p=0.009) and velocity (p=0.013) which are significant. **Fig.12.**

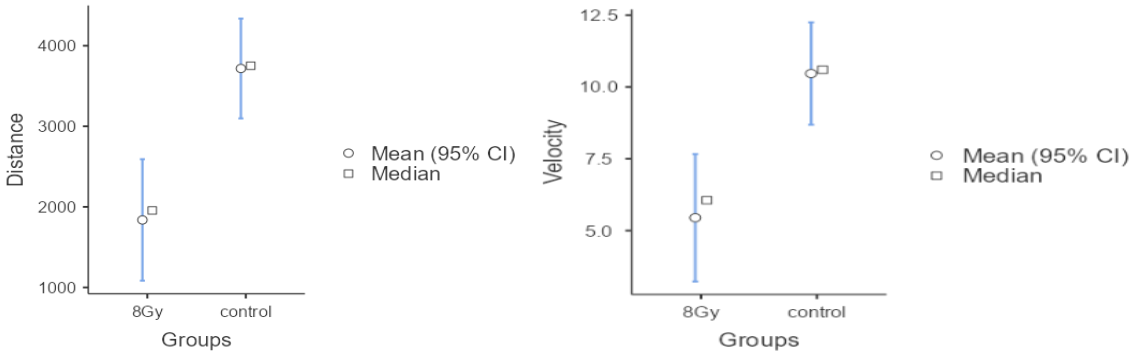


Fig.12. Showing 8Gy and control groups vs distance and velocity.

- We used Student's test for independent Samples T-Test to compare between 4Gy and control groups of SARRP experiment and it was observed that the distance (p=0.676) and velocity (p= 0.669). **Fig.13.**

Plots

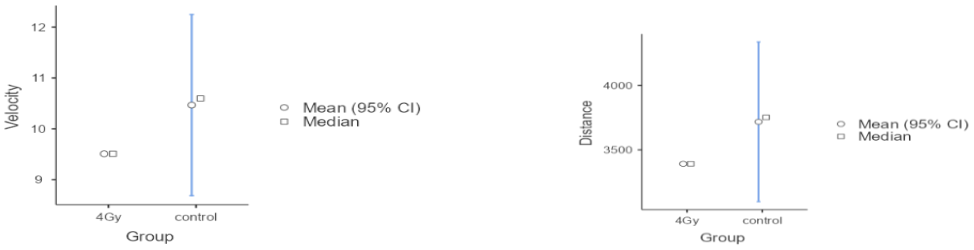


Fig.13. showing 4Gy and control groups vs distance and velocity.

- We used Normality and Student's tests to analyze the distance moved and the velocity mean of the irradiated groups. In **Fig.12**. (8Gy) shows significant difference of short distance moved by the irradiated (8Gy) rats and lower velocity than of the control group which refers to the damage of radiation on the brain function and behavior of the irradiated rats. On the other hand, in **Fig.13**. (4Gy) shows no significant difference.

(a) **(b)**

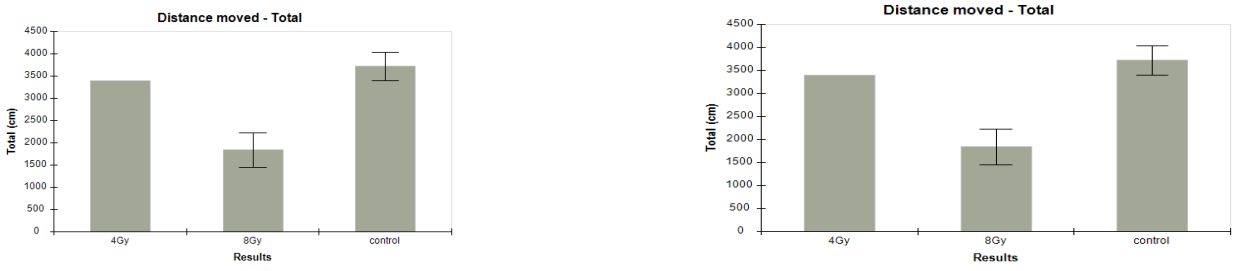


Fig.14: (a) Total distance moved during the experiment for the whole groups. **(b)**Mean velocities for the whole experiment.

Histological analysis:

- Fiji software was used to examine the histopathological images of Myelin. We used the binary pixel to figure out the Myelin sheath and calculate the % area of myelin distribution.
- Regarding statistical analysis of the histological study, we used the Normality test, Student's, and Mann-Whitney tests to compare 8Gy and control groups. In **Fig.15**,It's observed that % Area of Myelin is less in the 8Gy group than in the control group which indicates the effect of radiation on the SARRP rats, and it is obvious that there is demyelination.
- **Fig.16.** and **Fig.18.** show the Luxol Fast Blue (LFB) stained Myelin of SARRP control and 8Gy groups, respectively. **Fig.17.** and **Fig.19.** show the ImageJ made by Fiji software to evaluate the % Area of Myelin in control and 8Gy groups respectively after using irradiation.
- When we used independent samples T-test (Student's T)and normality tests to compare between control and 8 Gy groups in % area of myelin distribution (p= 0.086) and when we used Mann-Whitney (p=0.081) Normality test Shapiro-Wilk (p= 0.561) for % area of Myelin distribution.

Plot

% Area of Myelin

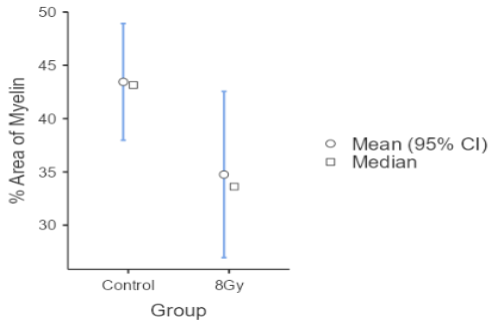


Fig.15: %Area of Myelin in control and 8Gy groups of SARRP.

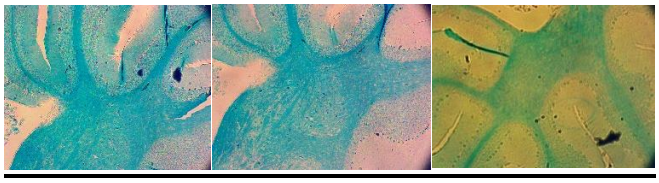


Fig.16: Luxol Fast Blue stained Myelin of SARRP control group.

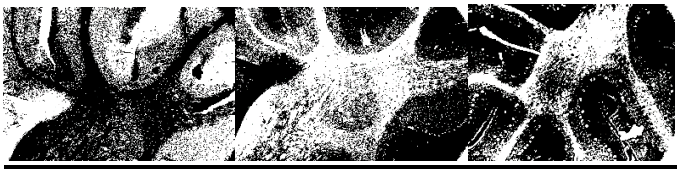


Fig.17: Fiji images of Myelin distribution in SARRP control group.

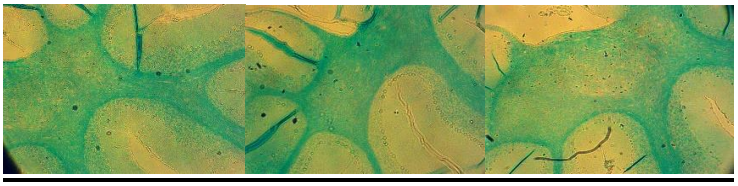


Fig.18: Luxol Fast Blue stained Myelin of SARRP 8Gy group.

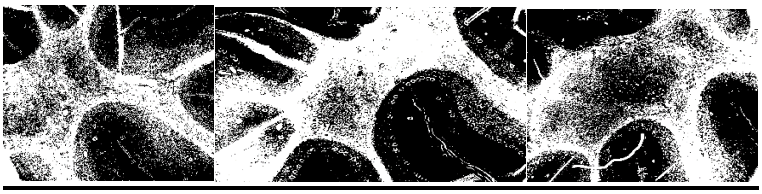


Fig.19: Fiji images of Myelin distribution in SARRP 8Gy group.

Conclusion

In conclusion, in this internship we investigated the efficacy of x-ray and proton irradiation as a model of radiotherapy for brain and pituitary gland. We used several techniques and tests to help us accomplish our goal of this study such as open field test, histopathological investigations and different software such as JAMOVI 2.5.6 software for statistical analysis going through the appropriate tests for our work and FIJI software for analyzing % area of myelin distribution in the histological samples. We figured out some interesting results regarding these techniques. The 8Gy irradiated rats in SARRP experiment suffered from anxiety ($p=0.031$) and this was observed through frequent entries of the central zone. In the histopathological analysis, it was observed that the %area of myelin distribution in the 4Gy irradiated rats was less than the control group which indicates the adverse effect of irradiation on the myelin sheath of this group. On the other hand, the pituitary gland weight in the 4Gy irradiated rats was less than this of the control group ($p=0.058$). These findings show the efficacy of radiation on the brain and pituitary gland and give us a hint to think about the future directions in this research scope.

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