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Irradiation of *Drosophila melanogaster* Leads to Increased Autophagy in Multiple Adult Tissues

Terrence M Trinca[1], Bilal R Malik[2,*]

[1]School of Biochemistry, University of Bristol, Bristol, BS8 1TD, United Kingdom

[2]Biomedical and Forensic Sciences, School of Human Sciences, University of Derby, Derby DE22 1GB, United Kingdom

[*]Correspondence should be addressed to Bilal R Malik, b.malik@derby.ac.uk

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Abstract

A common source of tissue toxicity is ionizing radiation to which humans can be exposed in a variety of ways including environmental contamination, radiotherapy, space and aviation travel. Radiotherapy is the most common method used to treat majority of cancers. However, the irradiation of patients can lead to many negative secondary effects causing irreparable internal organ damage due to its genotoxic effects and disruption of oxidative state of cells which, if left unresolved, can lead to cell death. Cells use autophagy as a homeostatic mechanism to remove debris and damaged organelle. However, it is not known whether autophagy is affected by radiation. *Drosophila melanogaster* has become one of the most trusted model organisms to study human disease and various biological pathways. Here we used Gamma radiation exposure to determine, for the first time, whether radiation influences autophagy in two different tissues in *Drosophila*: the midgut and the brain.

Keywords: Radiation, Autophagy, Cancer, Gut, Redox, Oxidative stress

Introduction

Maintaining a healthy cellular state is crucial for the survival of any animal, and so complex cellular protective mechanisms have evolved to defend organisms against the various toxic substances that they constantly encounter throughout their lifespan. Some of these substances are used in medicine and so cannot be avoided and a classic example is ionising radiation. While the genotoxic effects of radiation have been long known and well understood, how exactly the cell dynamically responds to irradiation has yet to be fully understood [1].

Radiations have been used in medicine for over a century with varied application [2,3]. In modern medicine, radiation in the form of Radiotherapy (RT) is used in the treatment of cancers, and has been estimated to be administered to approximately 52% of all cancer patients due to its curative potential [4]. The problem with using radiation to treat tumours is that it is non-specific and it can lead to the overspill of toxic byproducts of radiation damage to bystander non-irradiated tissues [5-7]. This is unavoidable and depending on the area of the body, irradiation can lead to a range of short and long-term side effects that include nausea and cognitive decline [8-11]. Once a cell has been exposed to high doses of radiation, which are required for treatment of cancers, they respond by activation of a signalling cascade either leading to cell cycle arrest or apoptosis which depends on the tumour suppressor protein p53. In mice, for example, whole body irradiation has been shown to activate p53 in a tissue specific manner. P53 null mice show resistance to radiation mediated apoptosis of cryptic epithelial cells [12,13].

Radiation reacts with water (radiolysis), producing ROS, which can interact with macromolecules, such as lipids, proteins and DNA, inducing oxidative damage and stress [14]. Radiation-induced oxidative damage has been extensively studied in humans, and ROS imbalances have been shown to persist after treatment [15]. From looking at the *Drosophila* research, it is clear that radiation induces oxidative stress, at least short-term. Genes such as *GstT4* and *GstD1* involved in oxidative

metabolism have been shown to have increased expression *post* irradiation [16,17]. ROS imbalances is also known to cause autophagy modulation through mTOR-AMPK pathway [18].

Once the cells have incurred sufficient subcellular organelle or protein damage it may respond by activation of autophagy to degrade and remove damaged material. Autophagy however requires functional lysosomes which contain the hydrolases on which autophagy depends. The damaged substrates are recognised by the cell and tagged for degradation. Failure to do so can result in the cell building up damaged materials such as oxidised proteins and lipids which can then interfere with the normal functioning of the cell [19].

Drosophila is a useful model system employed to understand the toxic effects of radiation in a living whole animal model [15,20].

While it is known that exposure to radiation leads to activation of the apoptotic pathway in *Drosophila* [21], nothing is known about how radiations affect autophagy, one of the cells most common response to toxins and damage [22,23]. Here we used *Drosophila melanogaster* as a model system to evaluate the effect of radiation exposure on autophagy.

Materials and Methods

Drosophila stocks and husbandry

Drosophila were raised at 25°C on standard cornmeal/molasses/agar media. The following *Drosophila* stocks were used: *uas-mCherry::Atg8* (gift from T. Neufeld), *GMR61G12-Gal4* (Flylight, Jenelia Farm), *Myo1A-Gal4* (gift from J. de Navascués).

Ionizing radiation exposure

3 to 6-day old flies were collected in vials containing cornmeal medium. A group of these was exposed to gamma irradiation using a cesium-137 γ-ray irradiator for a total of 150 Gy, administered at 0.43Gy/min. Control group was kept in the same room for the duration of gamma ray exposure.

Dissections, imaging and analysis

The flies, expressing uas-mCherry::Atg8 and uas-mCD8::GFP driven by GMR61G12-Gal4, were dissected as described previously [24]. Briefly, fly brains were dissected from 3 to 6-day old flies in cold PBS and fixed in 4% paraformaldehyde for 30 min. The tissue were then washed three times with PBST (10 min each), mounted in vectashield and stored at 4°C until imaged. Images were captured using Zeiss Spinning Disc Confocal (Cell Observer) microscope and a 63X oil immersion objective (numerical aperture 1.3).

Midguts were dissected from 3 to 6-day old flies directly into ice-cold PBS, fixed in a formalin (4%) and heptane solution for 15 min. Tissue was permeabilised using methanol (100%) for 15 min. Permeabilised tissue was then blocked 3x for 15 min each using PBS containing triton-X 100 (0.1%) and bovine serum albumin (BSA). Tissue was stained with the primary antibody Rabbit anti-RFP (Takara 632496) overnight (~16 hrs) at 4°C with mild rocking, followed by washing in PBT (3x rinses and 3x washes). Tissue was stained with the secondary antibody Donkey anti-Rabbit-A594 (Thermo Scientific A21207) for 2 hr at room temperature with mild rocking. DNA was stained with Hoescht at 1:5,000 (Sigma Aldrich B2261, stock solution at 10 mg/ml) which was added alongside secondary antibodies. Tissue was washed with PBST and mounted in home-made mounting medium (Glycerol: PBS 80:20 with added propyl gallate 4%). Confocal stacks were obtained in a Zeiss LSM 710 with an EC Plan16 Neofluar 40X and 63X oil immersion objective (numerical aperture 1.3). All stack positions were acquired in the posterior midgut. Image processing was done using FIJI [25] and statistical analysis was performed using Graphpad (PRISM 9.0).

Results

Radiation treatment leads to increased autophagy in the midgut

To assess the effect of gamma irradiation on the gut, we exposed a group of flies expressing mCherry-tagged Atg8 in midgut enterocytes (*Myo1D-Gal4>uas-mCherry::Atg8*). 150 Gy was chosen as this was similar to previously reported sub-lethal dosages of radiation to freshly eclosed *Drosophila* [26]. Dissections were performed 1 day *post* irradiation and no lethality or any other gross defects in the radiation-exposed flies were observed at this point. Upon irradiation, an increase in Atg8 was observed in the majority of midgut enterocytes and this was seen for the entire length of the midgut (**Figure 1A**). Individual cells had clear and multiple cytoplasmic puncta of Atg8 that had developed within 1 day of irradiation (**Figures 1B & 1C**). These puncta are likely to be autophagosomes which are clearing oxidised material resulted from irradiation [27]. Whole tissue Atg8 signal was quantified and determined to be significantly (2-fold) higher in irradiated compared to non-irradiated midguts (Unpaired t-test, p=0.008) (**Figure 1D**). We also observed a change in nuclear DNA reported by DAPI stain, but this was not quantified. This is consistent with previous reports of DNA damage caused by gamma irradiation [28].

Effect of radiation on the brain

To determine the effect of irradiation on adult fly brain we generated flies expressing mCherry-tagged *Atg8* in a small subset of neurons In the central brain which normally express pdf (*GMR61G12-Gal4>uas-mCherry::Atg8*). These neurons are relatively much bigger and could be easily imaged. This way we were able to focus on a small subset of neurons and look for changes in the mCherry signal within individual neurons. In comparison to the controls, we observed a significant increase in the Atg8 levels (Unpaired t-test, test, $p<0.0001$) indicating an increase in autophagy levels (**Figure 2**).

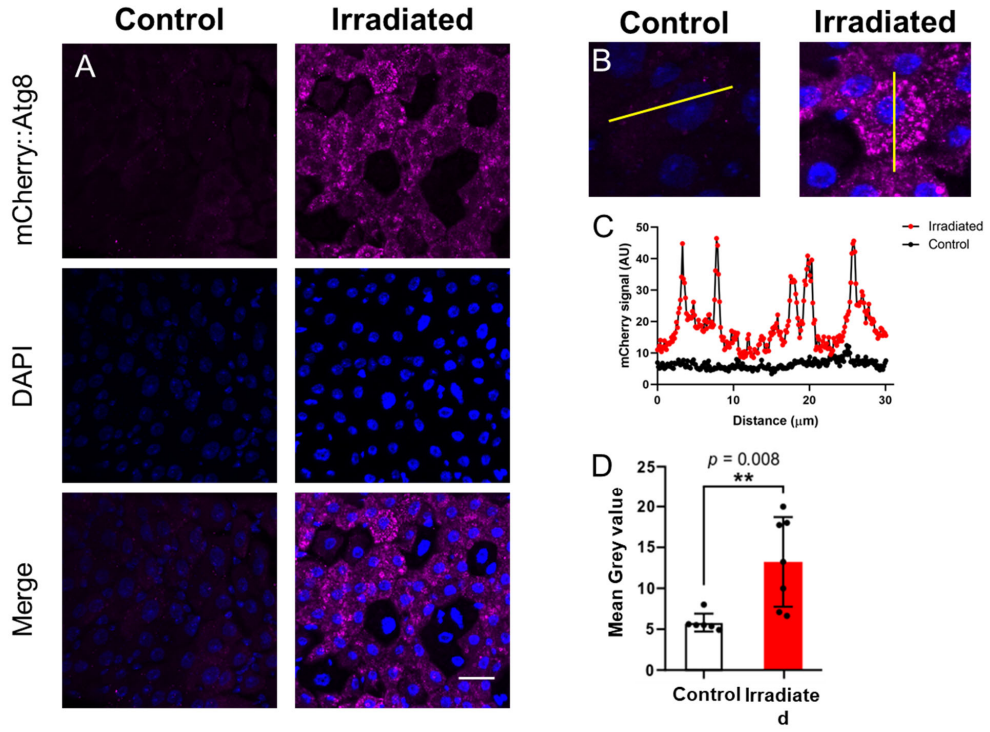

Figure 1: Effects of irradiation on midgut autophagy. Representative maximum projections of irradiated and control *Drosophila* midgut **(A)**. Scale bar is 20 microns. Digitally magnified close-ups of cells **(B)**, yellow line represents sampling path for panel **C**. mCherry intensity value per pixel along paths that were drawn in panel **C**. Whole-tissue measurements were taken of average mCherry signal/pixel **(D)** (t-test, n≥8).

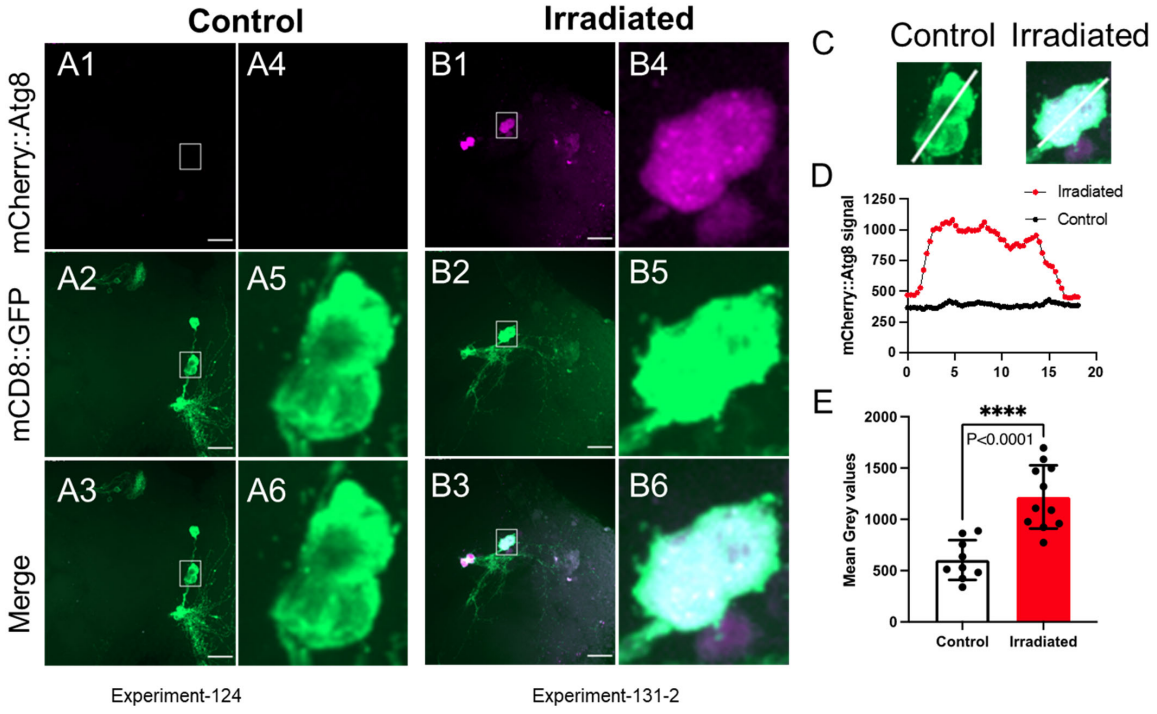

Figure 2: Autophagy is increased in response to Gamma irradiation. In comparison to control brains **(A)** the brains from irradiated flies **(B)** show increased neuronal levels of autophagy marker Atg8 **(B)**. Scale bar is 10 microns. Representative images of neurons **(C)**, solid lines indicate sampling path for mCherry signal sampling presented in panel **D**. Mean grey value of mCherry signal in control and irradiated fly neurons **(E)** (t-test, n≥8).

Discussion

Gamma irradiation is used with curative intent for the treatment of various cancers. The effect of these ionising radiations on different tissues and their radiosensitivity is an important factor which could help determine the optimum dosage for a given patient. The intracellular damage caused by repetitive exposure to such radiation poses a threat to patient's health who are already weakened by the impact of the disease. While the toxicity of the radiation can vary considerably from one patient to another, having an insight of their impact on the cellular mechanisms can help design strategies which can be in sync with radiation treatment to minimise the negative impact of radiations might have otherwise.

Cellular response to oxidative stress includes a waste-clearance mechanism, known as autophagy, which depends on a cascade of events leading to isolation of the damaged subcellular substrates and their degradation by the lysosomal enzymes. The effect of gamma radiations on this mechanism, so crucial for survival from cellular damage, is yet unknown. We show that cellular autophagy is significantly increased in response to gamma irradiation in two distinct types of tissues in young *Drosophila*. While the cellular morphology and the gut intactness in the time scale of our experiment was unaffected, the increase in autophagy might indicate an early response to the gamma radiations. The consequence of increased autophagy can be a determining factor for cellular survival. With significantly high damage to the cellular cytoplasm and DNA, autophagy mechanism could be overpowered leading to cell death. Since the neurons in the brain have several supporting tissues such as microglia and oligodendrocytes which help clear some of the secreted waste materials produced by stressed neurons it is pertinent to determine the impact of gamma radiation on these. In addition, to better understand the consequence of increased autophagy and whether the increased levels of autophagy actually represent a blockage of autophagic flux or a stimulation of autophagy requires further investigations. Similarly, the long-term response of radiation exposure on gut will require further experiments.

Future research to evaluate the effects of radiation should involve investigation of how different tissues respond to similar doses of radiation. The role of p53 has been shown to be crucial in response of gut epithelia to radiation [12,13]. Further understanding how this response compares between tissues would also help understand the dosage equivalence of radiation between tissues. A transcriptomic study comparing the responses of the different tissues to radiation and transcriptome level would be insightful in this regard.

Finally, extending these studies to human tissues will provide valuable insight and further validation of findings in model animals. *Drosophila* has proved valuable in understanding how radiation affect different tissues and the genetics involved in the response to radiation hinting towards different response by different tissues to radiation [26,29]. This report has shown that two completely different tissues of *Drosophila* develop the same molecular response to a fixed dosage of radiation suggesting molecular equivalence of the response of tissues to radiation. It however remains to be established whether this response provides any protection from the damage from radiation or if the benefit of this response is different in different tissues.

Conclusions

The use of radiation to treat various types of cancers and the negative secondary effects often seen in patients highlights the importance of understanding the mechanisms involved in radiation response from various tissues. One of the questions to consider is whether all tissues respond in a similar manner to radiation. For the first time, this study determined the effects of gamma irradiation on autophagy in two completely different tissue types in Drosophila. We show that autophagy is similarly affected in both the gut and the brain in *Drosophila* suggesting a common mechanism downstream of gamma irradiation.

Statements & Declarations

Competing interests

The authors have no competing interests to disclose.

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Author contributions

BM conceptualised and designed the experiments. BM and TMT prepared materials, collected and analysed data and wrote the manuscript. Both authors read and approved the final manuscript.

Data availability

The data associated with this manuscript is available from the authors.

Ethics approval

No ethical approvals were needed for the work.

Consent to participate and publish

No human subjects were involved in this work and hence there was no need for consent.

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