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Review article

An investigation into neutron-induced bystander effects: How low can you go?

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A B S T R A C T

Neutron radiation is very harmful to both individual organisms and the environment. A n understanding of all aspects of both direct and indirect effects of radiation is necessary to accurately assess the risk of neutron radiation exposure. This review seeks to review current evidence in the literature for radiation-induced bystander effects and related effects attributable to neutron radiation. It also attempts to determine if the suggested evidence in the literature is sufficient to justify claims that neutron-based radiation can cause radiation-induced bystander effects. Lastly, the present paper suggests potential directions for future research concerning neutron radiation-induced bystander effects. Data was collected from studies investigating radiation-induced bystander effects and was used to mathematically generate pooled datasets and putative trends; this was done to potentially elucidate both the appearance of a conventional trend for radiation-induced bystander effects in studies using different types of radiation. Furthermore, literature review was used to compare studies utilizing similar tissue models to determine if neutron effects follow similar trends as those produced by electromagnetic radiation. We conclude that the current understanding of neutron-attributable radiation-induced bystander effects is incomplete. Various factors such as high gamma contamination during the irradiations, unestablished thresholds for gamma effects, different cell lines, energies, and different dose rates affected our ability to confirm a relationship between neutron irradiation and RIBE, particularly in low-dose regions below 100 mGy. It was determined through meta-analysis of the data that effects attributable to neutrons do seem to exist at higher doses, while gamma effects seem likely predominant at lower dose regions. Therefore, whether neutrons can induce bystander effects at lower doses remains unclear. Further research is required to confirm these findings and various recommendations are made to assist in this effort. With these recommendations, we hope that research conducted in the future will be better equipped to explore the indirect effects of neutron radiation as they pertain to biological and ecological phenomena.

1. Introduction

Exposure to neutron radiation is extremely hazardous to both living organisms and the environment. Neutrons are produced by both fission and nuclear reactions. As neutrons are produced in the generation of nuclear power, exposure to neutron radiation is a major risk factor for nuclear energy workers (Evans et al., 1979). Moreover, neutron radiation is also a hazard associated with the use of nuclear weapons and contributed to the doses received by atomic bomb survivors following the detonation of the “Fat Man” plutonium bomb over the city of Nagasaki (Little, 1997). Neutrons are also a known component of cosmic radiation (Libby, 1946). Because of the destructive effects of neutron radiation on humans and the environment, an accurate assessment of its impact on living organisms is essential to reduce the harm associated with exposure. Importantly, along with the direct effects of neutron radiation, the indirect effects of neutron radiation must also be taken into consideration when assessing risk. Of particular interest are Radiation-induced Bystander Effects (RIBE), as they appear to predominate in low-dose regions for effects observed as a result of exposure to other forms of radiation, particularly gamma radiation and alpha particle radiation (Mothersill and Seymour, 1998; Nagasawa and Little, 1992). A the major effects of neutron radiation of living organisms is summarized in Fig. 9.

RIBE have been an extensive area of research since their discovery

Abbreviations: NTE, Non-Targeted Effect; RIBE, Radiation-Induced Bystander Effect; TUNEL, Terminal dUTP Transferase-mediated Nick End-Labeling; ICCM, Irradiated Cell-Conditioned Medium

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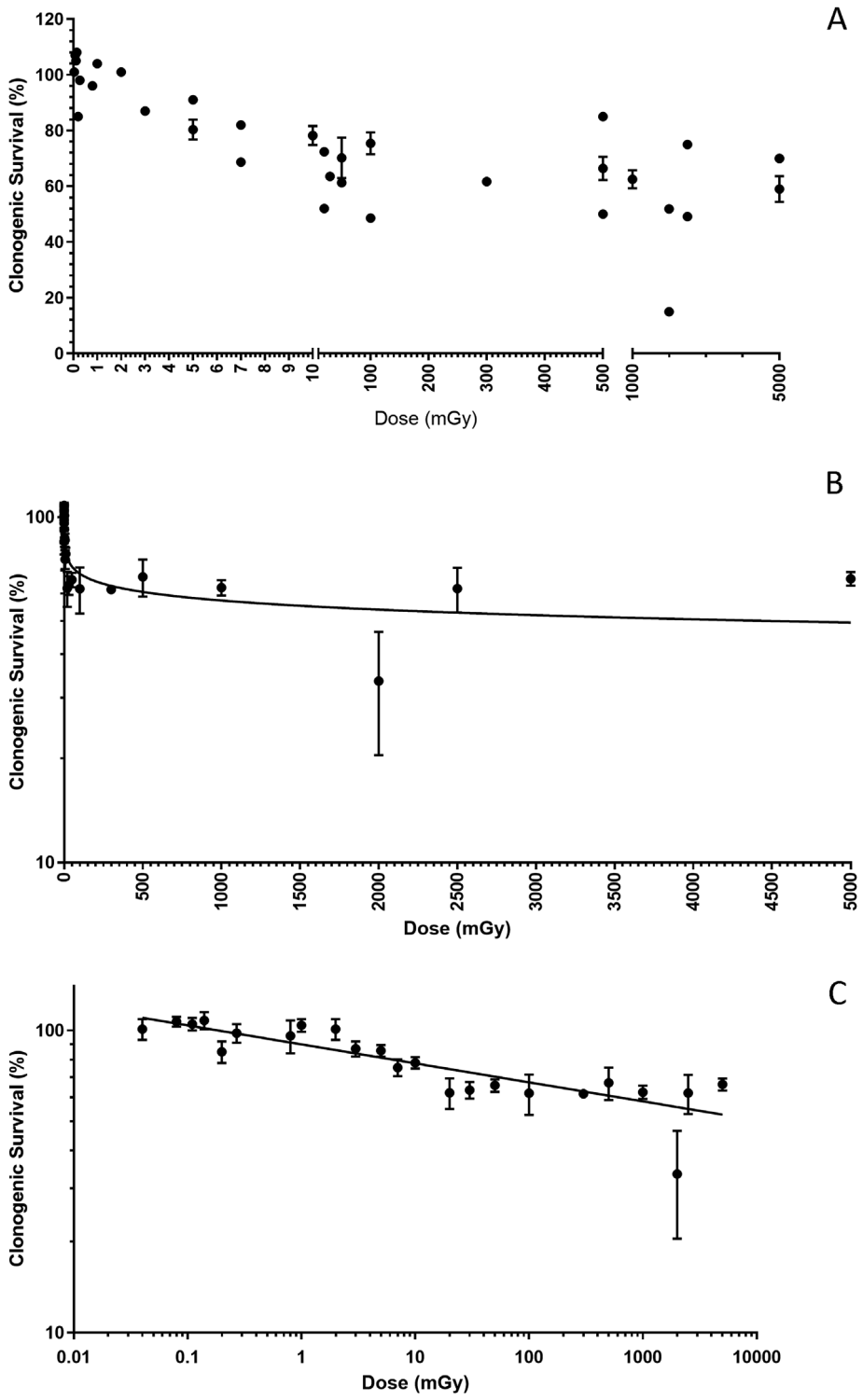

Fig. 1. A: Pooled from Table 1 showcasing the reduction in clonogenic survival of human keratinocytes exposed to ICCM of varying single doses ranging from 0.04 to 5000 mGy. B: Averaged reduction in survival for each administered dose fitted to a semi-logarithmic curve. C: Averaged data collected from Table 1 fitted to log-log curve.

in 1954 (Nagasawa and Little, 1992). Broadly defined as indirect effects of radiation observed in cells and tissues not exposed to radiation, relatively recent work in the field has led to a paradigm shift in the last few decades that has directed focus away from DNA-centric effects to coordinated signalling and tissue responses (Belyakov et al., 2000; Mothersill and Seymour, 2012; Mothersill et al., 2017a, 2017b). RIBE research has been garnering considerable interest since the discovery of medium-borne and gap-junction bystander signals in the late nineties (Seymour et al., 1997; Azzam et al., 1998). Alongside past and contemporary research into RIBE and other non-targeted effects of ionizing radiation (NTE) has come a better understanding of the broader effects of radiation exposure and an increased interest in potential applications of this newfound understanding in avenues of research such as radiation effects on the environment, dose and risk assessment for cancer therapy, and potential relevance in other human diseases (Mothersill and Seymour 2010, 2012; Nugent et al., 2010; Hei et al., 2011; Kryston

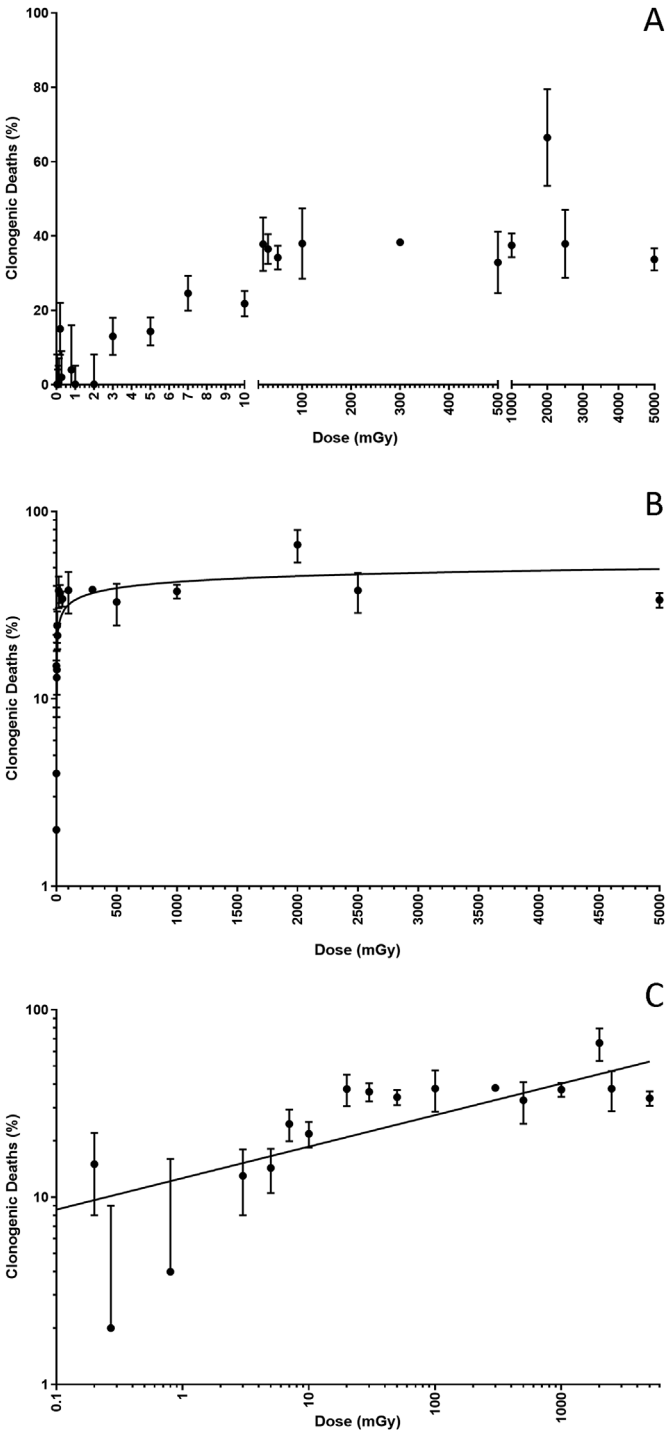

Fig. 2. Panel a: Data collected from Table 1 was used to calculate the percentage of clonogenic cell deaths for single doses of ICCM ranging from 0.04 to 5000 mGy. Panel b: Averaged increase in clonogenic deaths were fitted to a semi-log curve. Panel c: Averaged cell deaths were then fitted to a log-log curve.

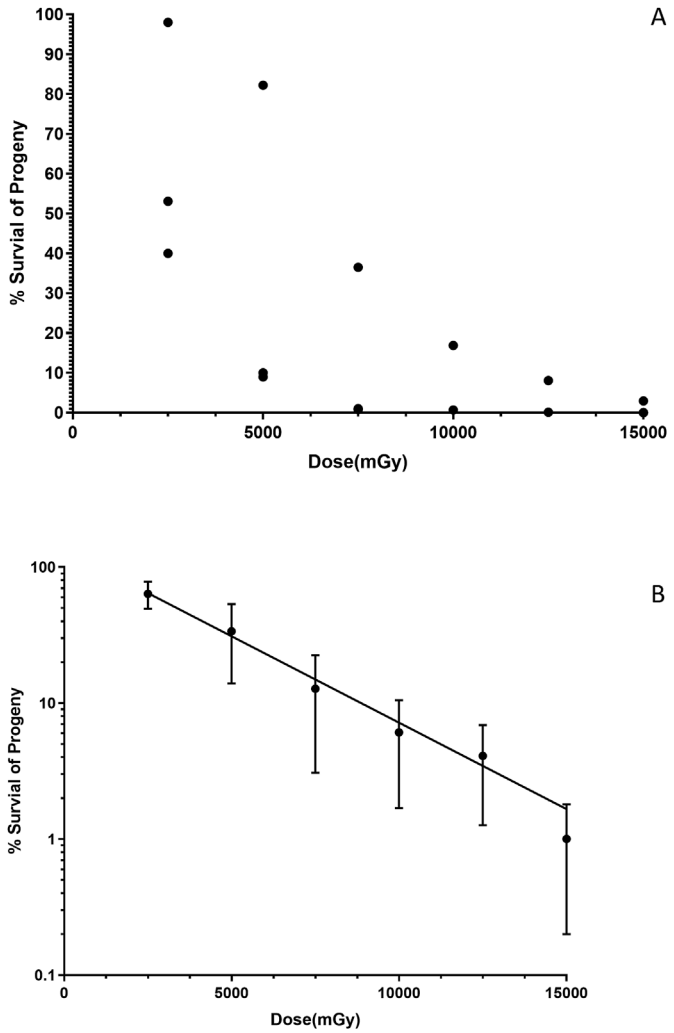

Fig. 3. Panel a: Pooled data from Table 2 demonstrating the reduction of survival for progeny of parent Chinese hamster ovarian cells, exposed to gamma doses ranging from 2500 to 15000 mGy. Panel b: Mean data from Table 2 fitted to a semi-logarithmic curve.

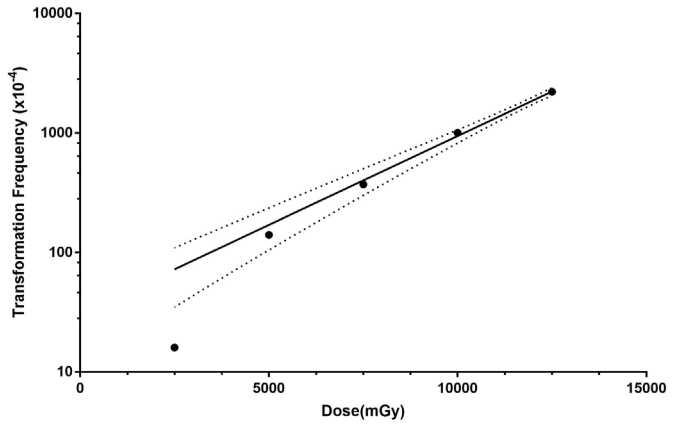

Fig. 4. Frequency of transformations observed in mouse embryonic fibroblasts fitted to a semi-logarithmic curve with 95% confidence intervals.

et al., 2011; Azzam et al., 2012, 2003; Rusin et al., 2018).

RIBE and NTE are thought to be the product of soluble-factor signalling, like gap-junction intercellular communication and calcium signalling, along with physical signals in the form of electromagnetic waves (Hei et al., 2008; Baskar, 2010; Le et al. 2017a, 2018, 2017b; Curtis et al., 2018). Furthermore, these effects have been observed in cells irradiated with either gamma or alpha-particle rays and few studies have connected these effects to neutron radiation; specifically, gap-junction intracellular communication, calcium signaling, and physical electromagnetic signals have thus far not yet been observed as an indirect effect of neutron irradiation. However, when also considering that many studies have used similar endpoints to conclude the same results in neutron studies, as reviewed in this paper, it is likely that RIBE and NTE resulting from neutron irradiation manifests similarly to

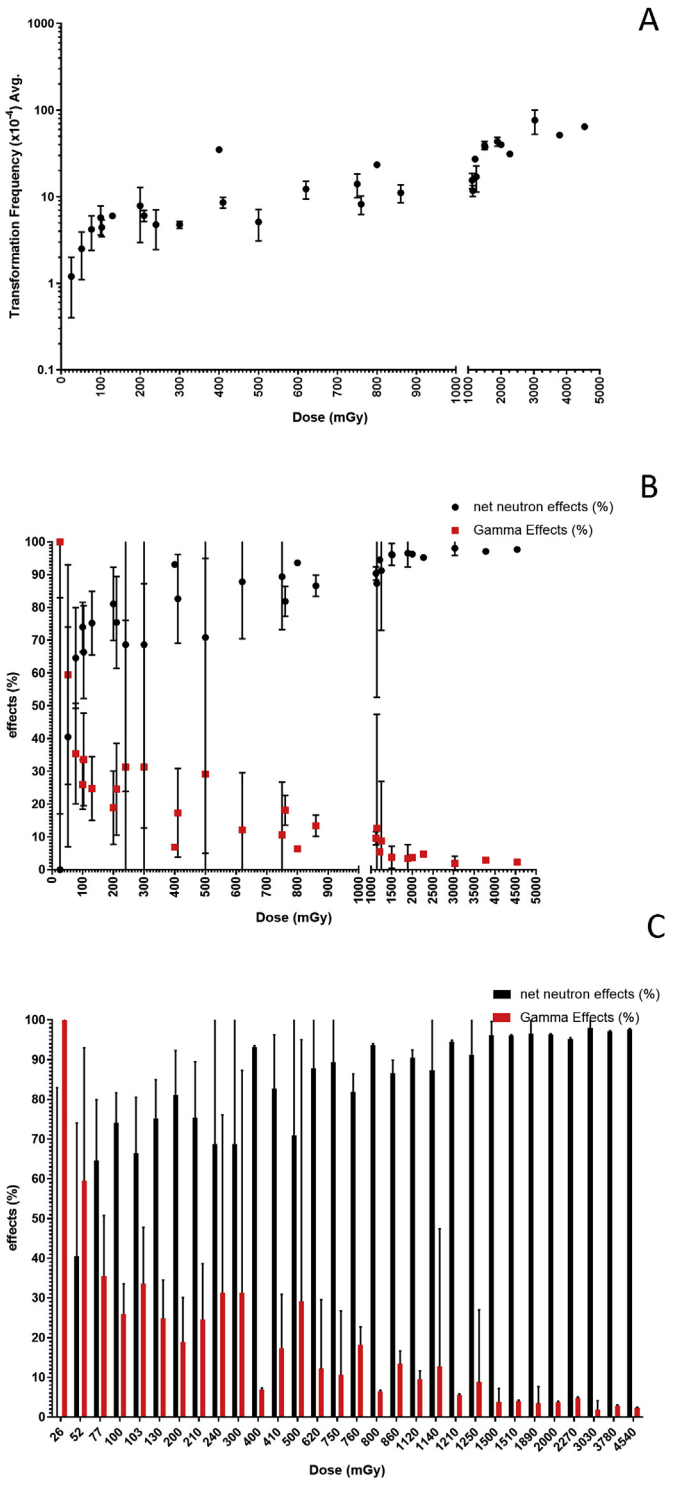

Fig. 5. Panel a: Selected data pooled from Table 3 displaying the occurrence of transformations in mouse embryonic fibroblast cells, subject to neutron doses ranging from 26 to 4540 mGy. Panel b,c: A comparison between percent neutron and gamma contributions to the overall effects.

RIBE and NTE resulting from gamma or alpha-particle irradiation. Various other NTE have also been the subject of research that are considered to be related to RIBE. Among these effects are genomic instability and lethal mutations due to radiation exposure (Mothersill and Seymour, 1997a; Bowler et al., 2006; Mothersill et al., 2017a). Observations of lethal mutations have been made numerous times both in the progeny of cells exposed to ionizing radiation and cells exposed to medium conditioned with irradiated cells (ICCM) (Seymour et al., 1986a,b; O'reilly et al., 1994; Mothersill and Seymour, 1997b; Seymour and Mothersill, 1997; Mothersill et al., 1998, 2000; Vo et al., 2017a). Several other NTE have also been observed in cells treated with ICCM, such as neoplastic transformation of normal cells (Hill et al., 1982, 1984a; 1984b, 1985; Jones et al., 1988; Miller et al., 1990; Hill and Zhu, 1991; Frankenberg-Schwager et al., 2009). Recently, an increased interest in low-dose radiation biology has emerged due to several studies demonstrating that RIBE predominates at doses below 100 mGy. Effects at these low doses are of particular interest because of potential applications in the protection of both man and the environment (Seymour and Mothersill, 2003; Mothersill et al., 2017b, 2018a). Some publications have discussed the fact that doses lower than 100 mGy are relevant to radioprotection and the various applications of radiation for human use, such as radiotherapy (Mothersill et al. in press).

A great number of endpoints have been used to quantify NTE. One such endpoint is cell survival, and is tested by the clonogenic survival assay developed by Puck and Marcus (1956). This assay measures the number of surviving colony forming units after a treatment, such as direct radiation exposure. While classically used to assay the effects of direct radiation exposure and toxic compounds, the method has been used for decades and has been established as a standard for quantifying RIBE and lethal mutations (Seymour and Mothersill, 1984; Mulgrew et al., 1985; Born and Trott, 1988; O'reilly and Mothersill, 1997; Seymour et al., 1997; Sawant et al., 2002; Dunne et al., 2003; Le et al., 2015a, 2015b; Vo et al., 2017a; Mothersill et al., 2018b). Various groups have also used apoptotic endpoints to assay NTE. Because NTE and RIBE have been established to cause apoptosis in reporter cells (Belyakov et al., 2002; Lyng et al., 2006a, 2006b; Vines et al., 2009; Singh et al., 2011; Smith et al., 2012; Widel et al., 2015), several papers have used endpoints such as mitochondrial viability, apoptosis-related gene expression, DNA damage, and micronuclei formation to quantify the reception of a bystander signal (Lyng et al., 2000; Belyakov et al., 2001; Azzam et al., 2002; Little et al., 2002; Ng et al., 2015; Rusin et al., 2019). Genomic instability in the progeny of irradiated cells has been assayed using chromosomal aberrations. Other endpoints include calcium fluxes, serotonin levels released into culture medium, and oxidative stress (Lyng et al., 2006a, 2006b; Shao et al., 2006; Pinho et al., 2012; Curtis et al., 2018). For the purposes of this study, these endpoints have been deemed as evidence of RIBE and NTE in "indirect exposure to radiation" studies, while they have also been observed in cells directly exposed to radiation.

Many of these effects have been observed following exposure to low electromagnetic radiation and more specifically have been observed with high and low linear energy transfer (LET) radiations. Many experiments conducted in the past that have found evidence for the existence of RIBE, have opted to use either gamma irradiation or alpha irradiation (Nagasawa and Little, 1992; Zhou et al., 2000; Mothersill et al. 2006, 2014; Ghandhi et al., 2008; Nugent et al., 2010; Hertel-Aas et al., 2011). Endpoints such as chromosomal instability have been attributed to both high LET (i.e alpha particles) and low LET (i.e. gamma rays, beta particles) radiation, along with a reduced clonogenic survival also being observed. Neutrons have a high LET and are known to be the one of the most damaging forms of radiation to humans and the environment (Ritter et al., 1977; Woloschak and Chang-Liu, 1990; Goodhead, 1999), yet the relationship between neutron irradiation, RIBE, and related effects has not entirely been validated. Many publications have either provided potential evidence for or attempted to dispute the existence of RIBE effects attributable to neutron radiation. Confounding factors such as gamma contamination of the radiation, cell line, and dose rates have caused inconsistencies in the literature. Gamma contamination is a phenomenon that is attributed to all neutron sources and is used to describe the contributing dose of radiation that is made up of electromagnetic radiation (Conger and Giles, 1950). Various other differences in the methodologies of various studies in the literature (e.g. TUNEL assay vs. clonogenic assay) can be used to

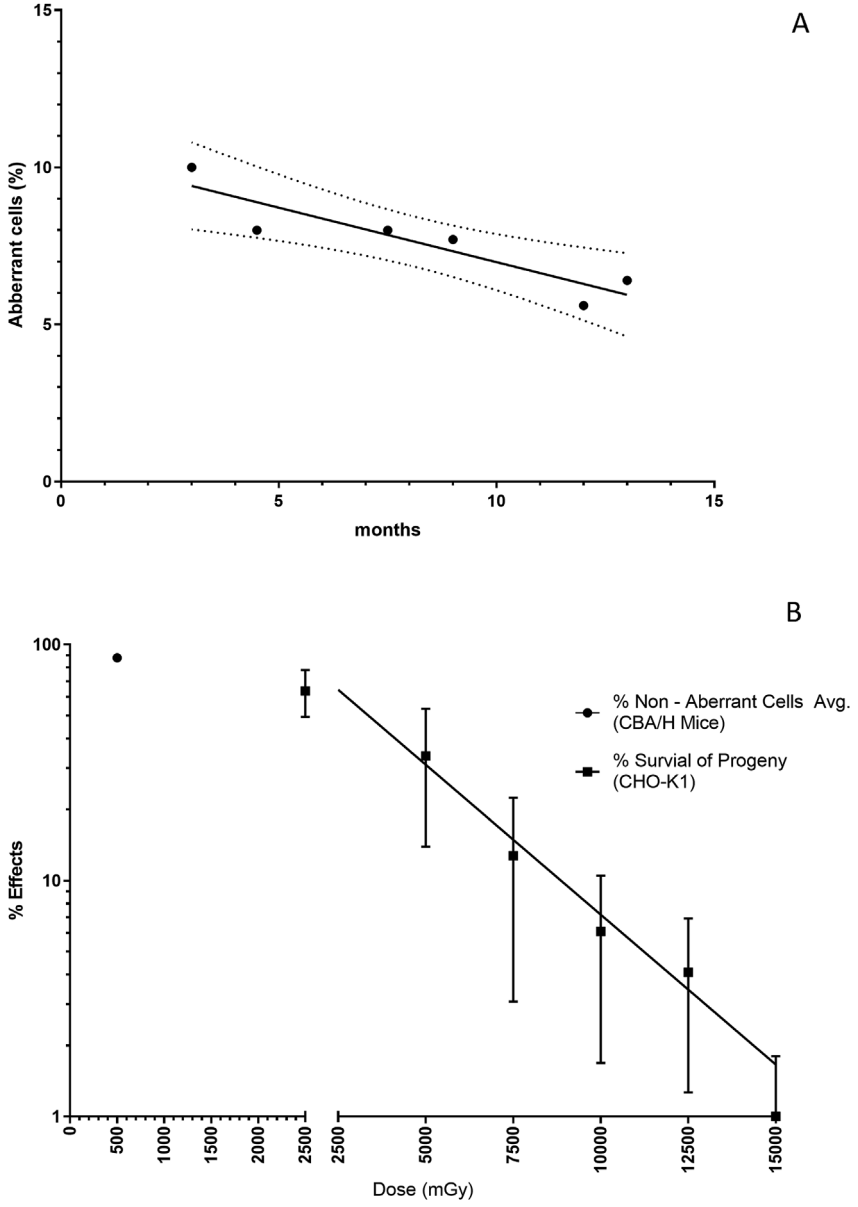

Fig. 6. Panel a: Percentage of aberrations in CBA/H mouse bone marrow cells over a total period of 13 months, post-neutron irradiation of 500 mGy fitted with 95% confidence intervals. Panel b: Data acquired in Table 3 is plotted from 2500 to 15000 mGy on the dose axis while the average percent of non-aberrant mouse bone marrow cells were plotted at 500 mGy.

potentially explain why some groups found putative evidence for neutron-induced RIBE and why some found potential evidence against neutron RIBE. The goals of this paper are chiefly to review the current evidence in the literature for RIBE and related effects attributable to neutron-based radiation and to determine if the suggested evidence is enough to justify claims that neutron-based radiation can cause RIBE. The present study uses a pooled dataset consisting of data from various publications studying RIBE due to both neutron and gamma irradiation. A comprehensive analysis of the literature was performed by plotting existing data on graphs and conducting various statistical tests to determine significance. Upon analyzing and reviewing this evidence, several key problematic features of neutron-based research became apparent. Furthermore, this goal was extended to outline these features and attempt to provide explanations for the different effects observed in the literature.

2. Methods

The present study incorporates datasets from different groups performing experiments under different conditions. There are various factors that cannot be controlled for in doing such a study, such as dose rates and cell lines. Attempts were made to control for these factors, however due to the limited data in the literature, observational comparisons were made between different aggregate data sets. Statistical analyses were performed by either direct comparisons of means or by determining the strength of trends for various plots. Ideally, a gamma dose threshold for observing these effects would have been established before these experiments to determine their validity with respect to effects directly attributable to neutron exposure. However, because this was not established in the majority of cases, analyses that were conducted in this study should be viewed as a good first step in determining the net effect of neutrons on RIBE in addition to demonstrating the importance of accounting for factors such as dose thresholds in the future.

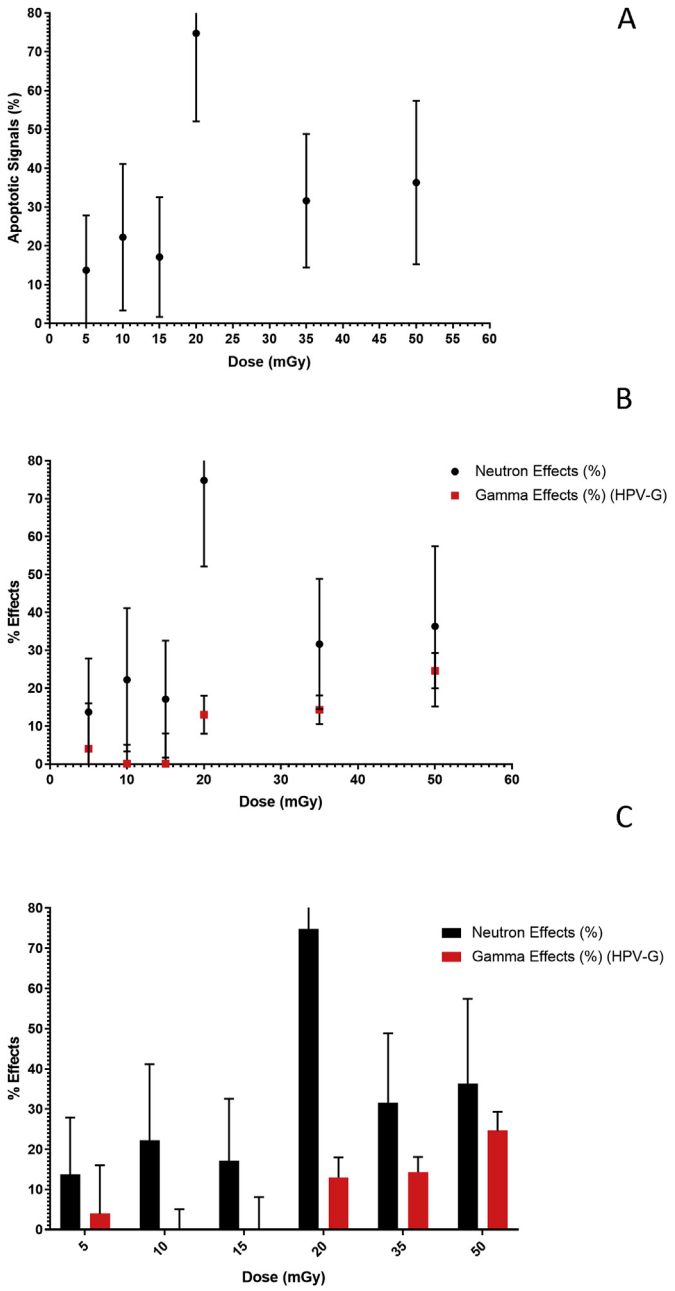

Fig. 7. Panel a: Apoptotic signals produced by zebrafish embryos after having received neutron doses ranging from 5 to 50 mGy (doses above 50 mGy were omitted; further discussed in the results section). Panel b,c: Net neutron and gamma effects plotted on the same axis for comparison with respective errors.

The meta-analysis was performed by first establishing a baseline gamma irradiated dataset, consisting of multiple data points collected from our lab, for the various endpoints tested. Multiple graphs compiled by the statistical analysis software Graphpad Prism 7 were performed to clearly demonstrate potential trends in both gamma and neutron datasets. Various logarithmic and semi-logarithmic functions of the form were then assigned to each $f(x) = \pm a*ln(x) \pm b$ respective gamma graph (although not explicitly mentioned in all cases) to verify already established trends that were observed when performing gamma-induced bystander experiments. Values a (slope) and b (y-intercept) are representative of the total observable effects (i.e. clonogenic survival, lethal mutations, neoplastic transformations etc.) and ultimately determined the overall trends shown. These equations were then applied to the neutron datasets, where applicable, to present a potential level of gamma contributions to the overall effects observed. For the baseline data collected, criteria such as cell line, dose rates, and doses determined which datasets to use to allow for a closer comparison between neutron and gamma effects. It is important to note that, due to variability between datasets, we were only able to apply one of the semi-logarithmic functions to the neutron dataset and only explicitly for the results shown in Fig. 5. Comparisons between the remaining datasets were carried out by plotting both neutron and gamma datasets on the same graph (Figs. 6 and 7).

Due to the various factors (i.e. dose rates and cell lines) and discrepancies between publications, the error analysis that was conducted is unique to each figure; it was performed for the most fitting representation of the statistical comparisons made. Generally, this comprised utilizing datasets where errors were explicitly given and included studies that did not explicitly show errors. The pooled datasets contained both explicit and implicit errors; we made use of the errors given and assumed no errors for those that did not explicitly report them. This was done to ensure the fairest evaluation of the datasets compiled for the respective figure. For the cases where explicit errors were completely absent, confidence intervals of 95% were employed as a substitute to provide information on the statistical behaviour of the respective dataset and to represent the goodness-of-fit for the non-linear regression. Special cases (i.e. where errors were calculated through basic error propagation) will be discussed further in the results section. All error bars displayed represent the Standard Error of the Mean (SEM).

3. Results

3.1. Data compiled from clonogenic survival experiments using gamma-irradiation and the HPV-G cell line

Fig. 1 represents the conventional trend that has been observed with the bystander effect and serves as a baseline when investigating potential trends for other NTE. By taking the average of the data collected from literature in Table 1, an overall decrease in survival occurs as directly irradiated cells receive increasing doses. Trendlines fitted to Fig. 1b (fitted with a semi-log curve) and 1c (fitted with log-log curve) are in accordance with our current understanding of RIBE and validate the data collected from this lab. Errors shown in Fig. 1c indicate a strong existing correlation due to the presence of small error bars and further supports the validity of this dataset. In this case where error bars are not explicitly shown, it can be assumed that its error is negligible (excluding the few data points that lacked error; refer to Table 1).

3.2. Data compiled for gamma radiation-induced clonogenic death experiments using the

Similarly, to Figs. 1 and 2 also complies with trends observed for the bystander effect, however it assesses clonogenic deaths. Obtained by taking the difference from 100% for the data in Fig. 1b and c, the appropriate error analysis was then implemented to acquire the SEM observed. As expected, an increase in reporter cell death is observed as a result of increasing dose. When observing Fig. 2c, the proportion of cell deaths beyond 20 mGy approach what resembles a plateau region. This is characteristic of the RIBE and further validates the baseline dataset being used. Data compiled for the survival of the progeny of CHO-K1 cells that have undergone lethal mutations due to gamma irradiation.

Fig. 3 was compiled from datasets collected in Table 2, with a similar trend to that shown in Fig. 1. However, due to the limited amount of literature available for CHO-K1 cell line, data points for lower doses were omitted. Fig. 3a lacks the presence of error bars as the datasets did not provide explicit definitions of the type of error calculations used, however the presented data was used to calculate the values observed in Fig. 3b and subsequently, their SEMs. Despite having a shortage of

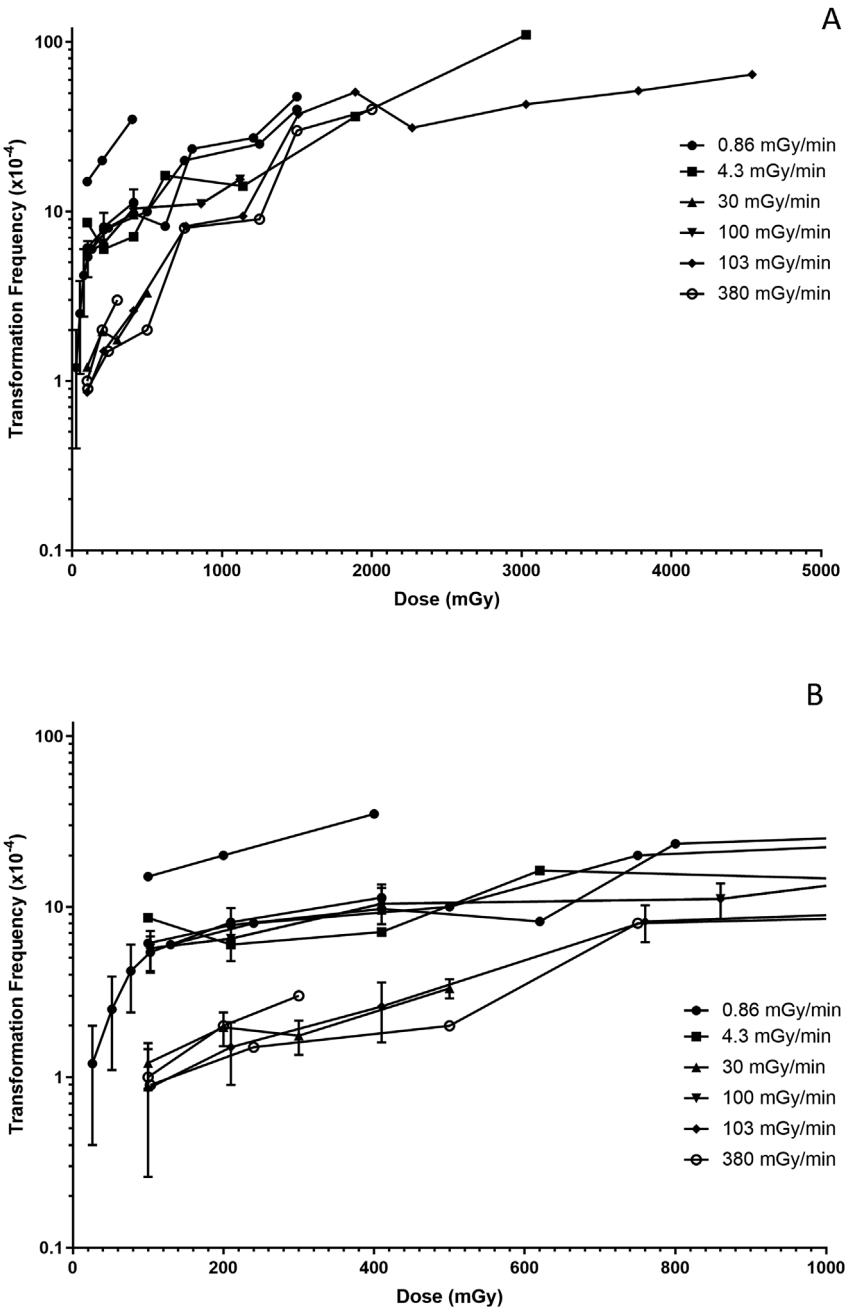

Fig. 8. Panel a,b: Varying dose rates from data in Table 3 were plotted to observe changes in transformation frequencies. For (b) a smaller dose range was used to magnify the lower dose regions.

data points, the mean data all lie within the trendline observed in Fig. 3b, indicative of a strong correlation at higher dose regions.

3.3. Data compiled for the frequency of transformations observed in C3H-10T1/2 cells irradiated with gamma radiation

Similar to Fig. 3, not many datasets were available for this combination of cell line and endpoint, which was the reason for the omission of the low dose regions. Data was obtained from Seymour and Mothersill (1997). The data demonstrated an increase in the frequency of transformations with increasing dose and is expressed as $f(x) = 0.0001487 \pm 0.000007 * ln(x) + 1.487 \pm 0.09$.

3.4. Data compiled for the frequency of transformations observed in C3H-10T1/2 cells irradiated with neutrons

Fig. 5a showcases a trend that is analogous to the observed trend in Fig. 4, while Fig. 5b and c both represent potential net neutron effects after taking the gamma contamination into consideration. When using the function obtained from Fig. 4, at a 4% contamination, it appears that gammas contribute a substantial amount to the overall observable effects at the lower dose regions; specifically at doses of 26 and 52 mGy. According to this function, gammas contribute 100% and 60% of the total effects observed, for doses of 26 mGy and 52 mGy respectively. As doses begin to increase, the overall contribution from gammas decrease. Data compiled for this Figure were obtained from Table 3. It is important to note that Fig. 5a consists of datasets that do not explicitly contain errors (refer to Table 3). Fig. 5b and c required utilizing the

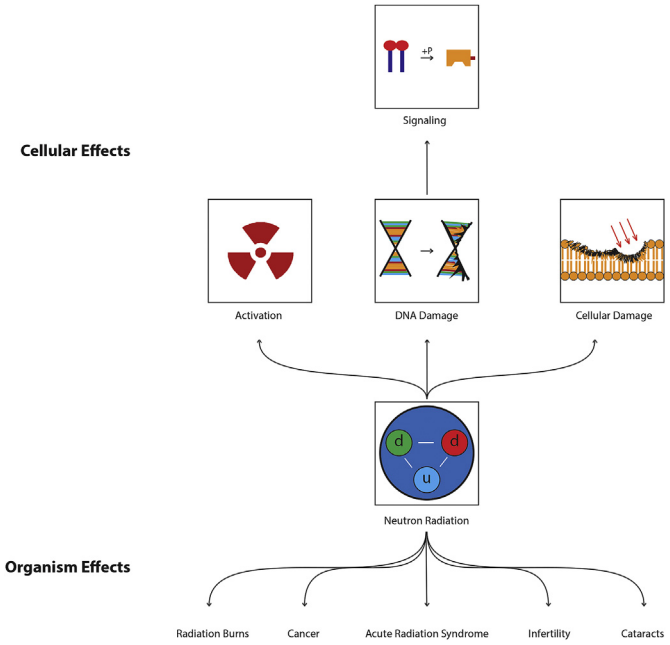

Fig. 9. A visual summary of the various effects that neutron radiation is known to have on living tissues and organisms.

Table 2
Pooled Data for Survival (%) of Chinese Hamster Ovarian progeny; progenitors exposed to varying gamma doses.

Reference	Source	Dose (mGy)	Dose rate (mGy/min)	Dose (mGy): Survival of Progeny (%)
(Seymour et al., 1986a, b)[a]	Cobalt 60 from St. Luke's Hospital	2500, 5000, 7500, 10,000, 12,500, 15,000,	1500	2500: 53.1 5000: 82.2 7500: 36.5 10,000: 16.9 12,500: 8.08 15,000: 2.97
(Mothersill and Seymour, 1993)[a]	Cobalt 60 from St. Luke's Hospital	2500, 5000, 7500, 10,000, 15,000,	1200	2500: 98 5000: 10 7500: 0.8 10,000: 0.7 15,000: 0.02
(Seymour and Mothersill, 1989)[a]	Cobalt 60 from St. Luke's Hospital	2500, 5000, 7500, 10,000, 12,500, 15,000,	1000	2500: 40.0 5000: 9.00 7500: 1.00 10,000: 0.70 12,500: 0.10 15,000: 0.02

[a] Indicates datasets that do not contain explicit errors.

errors from the equation stated above, along with error propagation, to obtain the respective errors. .

3.5. Data compiled for the proportion of lethal mutations observed in CBA/H mice cells irradiated with neutrons

Data was collected for Fig. 6 from the following publications: (Seymour and Mothersill, 1989; Watson et al., 2000). When observing

Table 1
Pooled Clonogenic Survival Curve Data for Human Keratinocytes after Exposure to ICCM of varying gamma doses.

Reference	Source	Dose (mGy)	Dose rate (mGy/min)	Dose (mGy): Survival (%) (± SEM)
Mothersill and Seymour (2002)	Cobalt-60 from St. Luke's Hospital	5–5000	1800	5: 80.3 (± 3.6) 10: 78.2 (± 3.4) 50: 70.2 (± 7.3) 100: 75.4 (± 3.9) 500: 66.4 (± 4.2) 1000: 62.5 (± 3.2) 5000: 59.0 (± 4.6)
(Mothersill and Seymour, 1998)[a]	Cobalt-60 from St. Luke's Hospital	500, 2500, 5000	500	500: 50 2500: 75 5000: 70
(Ryan et al., 2008)[a]	Cobalt-60 from Juravinski Cancer Centre	500, 5000	1700	500: 85 5000: 70
(Singh et al., 2011)[a]	Cobalt-60	20, 2000	500	20: 52 2000: 15
Seymour and Mothersill (2000)	Cobalt-60 from St. Luke's Hospital	10, 30, 50, 100, 300, 500, 2500, 5000	300	10: 68.7 (± 4.6) 30: 72.4 (± 3.1) 50: 63.5 (± 4.0) 100: 61.3 (± 5.9) 300: 48.6 (± 1.7) 500: 61.7 (± 0.69) 2500: 51.9 (± 2.9) 5000: 49.1 (± 1.3)
Liu et al. (2006)	KN Accelerator at McMaster University	0.04–7	0.7	0.04: 101 (± 8.0) 0.08: 107 (± 4.0) 0.11: 105 (± 5.0) 0.14: 108 (± 7.0) 0.20: 85 (± 7.0) 0.27: 98 (± 7.0) 0.80: 96 (± 12.0) 1.00: 104 (± 5.0) 2.00: 101 (± 8.0) 3.00: 87 (± 5.0) 5.00: 91 (± 2.0) 7.00: 82 (± 5.0)

[a] Indicates datasets that do not contain explicit errors.

Table 3
Pooled Data for Transformation Frequencies (TF) in C3H-10T1/2 exposed to varying neutron doses.

Reference	Source	Dose (mGy)	Dose rate (mGy/min)	Energy (MeV)	Y contamination (%)	Dose (mGy): TF (e-4) (± SEM)		
(Hill et al., 1982)[a]	JANUS at Argonne National Laboratory (ANL)	100–4540	0.86–380	0.85	4	0.86 mGy/min: 100: 6.1 210: 7.8 410: 9.7 620: 8.2 800: 23.4 1210: 27.2 1500: 47.6	4.3 mGy/min: 100: 8.6 210: 6.0 410: 7.1 620: 16.3 1140: 14.1 1890: 36.3 3030: 110.0	103–380 mGy/min: 100: 0.86 210: 1.4 410: 2.6 760: 8.2 1140: 9.4 1510: 37.5 1890: 50.6 2270: 31.2 3030: 42.9 3780: 51.5 4540: 64.4
Hill et al., (1984a) (1984b)	JANUS at ANL	26–760	0.86, 103, 380	0.85	4	0.86 mGy/min: 26: 1.2 (± 0.8) 52: 2.5 (± 1.4) 77: 4.2 (± 1.8) 103: 5.4 (± 1.3) 210: 8.1 (± 1.7) 410: 11.3 (± 2.2)	103 mGy/min: 100: 0.86 (± 0.6) 210: 1.5 (± 0.6)	380 mGy/min: 410: 2.6 (± 1.0) 760: 8.2 (± 2.0)
Hill et al. (1985)	JANUS at ANL	103–1120	100	0.85	4	103: 5.7 (± 1.5) 210: 6.5 (± 1.7) 410: 10.4 (± 2.5) 860: 11.1 (± 2.6) 1120: 15.5 (± 3.1)		
Miller et al. (1990)	RARAF of Columba University	100–500	30	5.9	N/A	100: 1.21 (± 0.4) 200: 1.96 (± 0.4) 300: 1.75 (± 0.4) 500: 3.32 (± 0.4)		
(Hill et al., 1948)[a]	JANUS at ANL	103–2000	0.86, 380	0.85	4	0.86 mGy/min: 130: 6 240: 8 500: 10 750: 20 1250: 25 1500: 40	380 mGy/min: 103: 0.9 240: 1.5 500: 2 750: 8 1250: 9 1500: 30 2000: 40	

[a] Indicates datasets that do not contain explicit errors.

Fig. 6a, an overall decrease in aberrant cells was observed as the length of time increased. This is similar to the trend observed in Fig. 3.

However this experiment only accounted for a single dose of 500 mGy. For Fig. 6b, the total aberrations over the duration of the experiment was used instead and due to this dataset testing for a different endpoint than in Fig. 3, the proportion of non-aberrant cells was used with attempts of creating a fairer comparison between neutron and gamma effects. Since the data for aberrant cells is collected across generations and passages, taking the proportion of non-aberrant cells is similar to looking at the percent survival of progeny. The endpoints are not exactly the same, therefore an in-depth comparison like the one observed in Fig. 5 could not be performed. Cell lines also differ, however both are mammalian which still allows for comparison between the datasets. The trend observed with the gamma data points appear to be strongly correlated when examining the error bars, with majority of data points falling within the trendline.

3.6. Data compiled for the production of apoptotic signals in zebrafish embryos that had undergone neutron irradiation

Fig. 7a compiled from Ng et al., (2015) and every paper listed in Table 1. All data from Table 1 was used to generate a baseline for gamma effects which was used to compare to Ng et al., (2015). Ng et al., (2015) used the terminal dUTP transferase-mediated nick end-labeling (TUNEL) assay to evaluate bystander effects in zebrafish embryos. The experiment claimed to have observed this phenomenon between a dose range of 20–50 mGy, with 14% gamma contamination. Data for doses above 50 mGy were determined to be insignificant which was the reason for their omission. No paper from our lab utilized the TUNEL assay, hence the clonogenic assay was used for comparison. Fig. 2 showcases trends observed with clonogenic deaths and was used since it is associated with apoptotic signals. At 14% gamma contamination, for neutron doses of 20, 35 and 50 mGy, gamma doses received by the embryos were 2.8, 4.9, and 7 mGy respectively. It is important to note that the SEM values indicated in Fig. 7a had to be calculated as error values were not explicitly provided by the authors; SEM values were calculated by using error propagation on the presented data.

3.7. Data compiled for the varying dose rates used for observing transformation frequencies in C3H-10T1/2 cells

Data collected for this figure were acquired from Table 3 and demonstrate an increase in observed frequencies when using lower dose rates. When observing Fig. 8b, dose rates ranging from 0.86 mGy/min to 100 mGy/min all appear to lie within transformation frequencies

ranging from 8 × 10˚-4 to 10 × 10˚-4. As the administered dose begins to increase more variations are observed with lower dose rates still dominating at higher dose regions. As mentioned for Fig. 5, certain datasets lacked explicit errors; consequently, these points were assumed to have no error.

4. Discussion

4.1. Review of evidence for neutron-induced RIBE

Baseline sampling of the gamma results appear to produce expected trends for RIBE experiments. The clonogenic data collected from papers utilizing HPV-G cells show a consistent logarithmic trend, indicating decreased survival with increasing dose (Fig. 1b and c). These results are corroborated by findings in each of the analyzed from our lab and others (Seymour et al., 1986a,b; Mothersill and Seymour 1993, 1998, 2002; Lyng et al., 1996; Seymour and Mothersill 1997, 2000; Mothersill et al. 1999, 2004; Ryan et al., 2008). While the confidence intervals are relatively narrow at lower doses, they appear to increase with increasing dose. This was determined to be most likely the result of increased variability with higher doses, which is an effect that has been observed in RIBE experiments using electromagnetic radiation outside of our lab and across different cell lines (Prise, 1998; Facoetti et al., 2006; O'Dowd et al., 2006). This effect is particularly apparent in Fig. 1a. This may be a result of varying dose rates between the higher and lower doses in the papers listed above. Similar expected trends were observed in gamma experiments using CHO-K1 cells in lethal mutation assays; specifically, an increased dose led to increased cell death at a fixed generation (Seymour and Mothersill 1989, 1997; Mothersill et al., 2002; Maguire et al., 2007; Singh et al., 2011). Transformation frequency increased with increasing dose, which was also an expected trend according to the literature (Terzaghi and Little, 1976; Lewis et al., 2001; Sawant et al., 2001; Ko et al., 2006), however some evidence does exist that low doses can reduce neoplastic transformation below spontaneous rates (Azzam et al., 1996; Redpath and Antoniono, 1998). The use of these graphs to determine the contribution of gamma radiation to neutron effects was therefore justified.

After analyzing the pooled data sets collected, it was determined that there exists evidence for RIBE inducible by neutrons. Fig. 5 represents transformation frequency data for the same cell line in both gamma- and neutron-based experiments. At lower doses, it is apparent that gamma rays contribute a substantial amount to the effects observed. However, at higher doses, gamma effects cannot account for the total effects observed. This observation at low doses is most likely due to high levels of gamma contamination, which is examined later in this discussion. However, these graphs do provide substantial evidence for the existence of NTE attributable to neutrons in higher dose regions; the effects of neutrons at lower doses however appears to remain undetermined. Fig. 7 also presents potential evidence for neutron induced apoptotic signal production. The percentage of contribution by neutrons appears to be high, especially at lower doses. However, the lower doses before 20 mGy were found to be insignificant by the authors. At higher doses after 20 mGy, the contribution of gamma and neutron radiation appears to become more evenly distributed. Therefore, these data sets do provide potential evidence for the existence of neutron-induced RIBE at doses between 20 and 50 mGy. However, these results are not conclusive for reasons discussed further in Section 5.2; gamma contamination is a concern for these results, as it was reported by the group to be 14%.

Some findings in the literature have demonstrated no such RIBE after neutron irradiation. The datasets represented in Fig. 6 failed to provide evidence that neutron RIBE exists at a genomic instability endpoint. This is further expanded in Section 5.2. Irrespective of whether a research group used their findings to evidence or disprove RIBE in the context of neutron irradiation, several major problems in experimental methodology seem to permeate through the literature. These problems primarily stem from various factors that could have been controlled for, however also lie in particulars of different methodologies between research groups. Some groups claim to observe NTEs due to neutron irradiation but have extremely high gamma contamination levels which may be contributing a significant amount of the effects seen. Others display low gamma contamination levels but did not take into account the gamma threshold, or the lowest dose at which NTE are observed, for the irradiated cell line. The choice of cells or tissues used for the experiments also may play a significant role in determining whether or not significant results can be obtained. Lastly, there exists evidence that other factors such as dose rate may also have an appreciable effect on the magnitude of the effects observed. Outlining such issues may serve to assist future efforts in researching neutron RIBE and other NTE.

4.2. Gamma contamination and dose thresholds

The foremost problem with most papers attempting to determine if neutrons can induce RIBE and associated effects is that the gamma contamination is not taken into account, and if it is taken into account, the associated gamma threshold for the cell line is not further investigated. If a cell line has a low enough gamma threshold after which RIBE are detectable, gamma radiation might still be contributing a significant amount to the observed effects irrespective of how low the contamination levels are. This is an issue that is particular to researching RIBE because many papers have described bystander effects both *in vitro* and *in vivo* using gamma radiation at various doses.

Many of the papers reviewed had issues with having very high gamma contamination during their neutron irradiations and not accounting for the dose threshold for the cell line being used. Among the papers that accounted for gamma contamination properly, a paper from Liu et al. (2006) described a medium transfer experiment using a human keratinocyte cell line and concluded its gamma threshold to be 2 mGy. Even though the neutron beam had a gamma contamination of 3%, a reasonably low contamination level, the authors established a gamma dose threshold after which RIBE were observed. The gamma doses in the neutron irradiations were kept below the threshold, and therefore it would be reasonable to assume that any observed effects could be attributed to neutrons. However, the study found no RIBE attributable to neutrons, while treatment with gamma rays above the dose threshold produced RIBE. An experiment from Frankenberg-Schwager et al. (2006) using hybrid normal human fibroblasts found that neutron exposure resulted in neoplastic transformation of the cells after a set number of generations. While not classically considered an RIBE endpoint, neoplastic transformation is closely linked to the phenomenon of genomic instability, which has been used as an endpoint for RIBE in the past (Wright, 1998; Little, 2000; Lewis et al., 2001; Morgan 2003a, 2003b). It is important to note however that this paper did not use any sort of medium transfer and only assayed direct descendants of irradiated cells. This was one paper that was found that had a gamma contamination of 1% during its irradiations, however a dose threshold was not established for this cell line. The researchers used higher doses of gamma radiation and found significant effects but did not establish after at what lower doses these effects might still occur. Therefore, even with a gamma contamination of 1%, it is still uncertain how much of what the authors saw was due to neutrons and how much was due to gamma rays. This problem is further exacerbated when analyzing the research published by Watson et al. (2000, 2001) investigating the neutron effects on chromosomal instability in mice. At gamma contamination levels of up to 33%, this becomes concerning when the directly irradiated cells received doses of 500 mGy (165 mGy attributable to gammas). However, when referring to Fig. 6b, neutron contributions appear to be miniscule despite having a high contamination level. Any observable neutron effect cannot be verified due to the baseline gamma dataset not consisting of points in the lower dose regions. The absence of any observable gamma effect could be the result

of the radiation being incapable of producing lethal mutations at low doses, providing a potential explanation as to why even with 33% contamination no observable effects were present. The study by Ng et al. (2015) also presented issues when the analysis was being conducted. When referring to the gamma threshold established by Liu et al. (2006) all gamma doses received by the embryos for the range used were above this threshold. Consequently, it is unreasonable to assume that gamma contributions can be elicited from overall effects and when observing Fig. 7c, gammas account for more than 50% of total appreciable effects at 50 mGy. Fig. 7a also exhibits significant variation in the data (observed by the presence of large error bars), raising questions on the validity of the conclusions drawn by the authors reviewed. Importantly, the compared cell lines and dose rates differ. Because of these issues in these papers, and others, the significance of the neutron data cannot be validated until a further investigation into effects of low dose gamma irradiation is conducted.

Transformation frequency of normal cells to tumorigenic cells was used as an endpoint for many papers describing the indirect effects of neutron radiation. Fig. 5 shows the pooled data set for these papers. The net contribution of neutron dose to transformation frequency was calculated using a separate data set as a reference for neutron effects. It was found that at lower doses, the contribution of neutron dose was virtually nonexistent, as gamma-radiation exposure at these doses where the neutron radiation is gamma contaminated can easily be used to explain the effects. Conversely, the gamma contribution appeared to decline with increasing dose while the neutron effects appeared to increase. This fact shows that perhaps neutron effects are not negligible for the transformation endpoint, particularly at higher doses. At doses below 100 mGy however, neutron effects remain undetermined. This can be further validated by observing Fig. 5b and c, where the overall decrease in the size of the error bars with increasing doses is observed, while at lower doses the statistical variance makes it difficult to draw meaningful conclusions. This is evidence that taking note of the gamma contamination alone is not justifiable when trying to distinguish between neutron and gamma effects; a comprehensive investigation into the respective gamma thresholds must be done in order to elucidate neutron-induced RIBE.

Our results seem to suggest that bystander effect contributions at low doses appear to result primarily from the gamma component of the radiation, and at high doses these effects appear to result primarily from the neutron component of the radiation. However, the evidence of specificity of either of these to the effects observed at relatively high or low doses is limited. It is possible that the entire biological effect from the neutron component increases with dose and the relative biological contribution of gamma decreases due to the comparatively large amount of energy deposited by the neutron component.5.3. Cell lines and tissue modelling.

A major issue in reviewing the data in the current literature is that different groups appear to use a wide range of different cell lines and a wide range of endpoints *in vitro, in vivo,* and *ex vivo*. Because RIBE and their associated effects are associated with signal transduction and intercellular signalling, different cells from different tissues, organs, and organisms most likely respond to the bystander signal differently (Nagasawa et al., 2002; Azzam et al., 2003; Zhou et al., 2005; Hei, 2006; Prise and O'sullivan 2009).

To demonstrate the differences that exist between tissues and different study models, two papers were compared utilizing cells from the same species. An experiment conducted by Ng et al. (2015) irradiated zebrafish embryos with neutrons and observed RIBE. The authors used apoptotic signals as their endpoint and accounted for gamma contamination. Experiments performed by Wang et al. (2011) did not observe RIBE when live zebrafish were irradiated with neutrons inside a tank. Tissue from the caudal fin was harvested from zebrafish irradiated *in vivo* and used to generate explant cultures, from which medium was harvested and used to treat HPV-G reporter cells. These reporter cells were assayed for clonogenic survival, and showed no reduction in survival. The two groups used very similar endpoints for their study, one being apoptotic signals detected by the TUNEL assay, and one being through clonogenic survival. The lack of effect observed in the latter study could be attributed to different tissue response to direct radiation exposure. While the zebrafish embryos appear capable of producing the bystander signal, tissue from the caudal fin does not seem to have the same capacity to generate bystander signals.

Observations of variance in either the production of signals from tissues or their response to the bystander signal has been observed in past experiments (Belyakov et al., 2003; Koturbash et al., 2007; Ilnytskyy et al., 2009; Rusin et al., 2019*)*. This could be due to a number of factors. Some cell lines could either be more sensitive to radiation and thus produce more signals compared to others (i.e CHO-K1 vs XRS-5) (Dahm-Daphi et al., 1993; Marangoni et al., 2000). Various cell lines and tissues have been found to be sufficiently insensitive to radiation exposure to generate a bystander signal or have been shown to be incapable of producing the bystander signal under various conditions (Mothersill et al., 2002). Some tissues or cell lines may not express the proteins required for either the generation of the signal or signal transduction, as is the case with p53 null cells (Mothersill et al., 2011; Kalanxhi and Dahle, 2012; Li et al., 2013; M. Le et al., 2017a, 2017b), along with having different gamma dose thresholds for RIBE. Radiosensitivity variance between cell lines and even species has been established in the literature as well (Mothersill et al., 2002). Moreover, different cell lines may also have different gamma thresholds for observing effects.

Such differences make it difficult to compare the various papers in the literature and to determine general trends between the studies. Despite knowing that there were bound to be discrepancies in the data because of the potential differences between cell lines and tissues, an attempt was made to compare the datasets collected from cells that were as similar to one another as possible. Clear trends can be observed in the data collected. Fig. 5 shows the transformation frequency data collected from C3H10T1/2 cells, a cell line created from mouse embryonic fibroblast cells (Hill et al., 1982). An increase in the overall neutron-dose contribution to the effects was observed with increasing dose, while gamma-dose contribution decreased. Apoptotic signal results from HPV-G cells, a human skin cell line, show a similar trend with increasing dose (Fig. 7) (Ng et al., 2015). Lethal mutation experiments conducted on both CHO-K1, a chinese hamster ovary cell line, and bone marrow explants from CBA-H mice were found to be the most similar publications for comparing the lethal mutation endpoint (Fig. 6) (Seymour and Mothersill, 1997; Rastogi et al., 2011) In this figure, a trend exists that appears to reduce the percent survival of cells with increasing dose. Figures showing gamma radiation results (Figs. 1–4) including ones representing clonogenic survival, death, lethal mutations, and transformation frequency with dose are shown using the same cell line, respectively.

For future experiments, a good starting point would probably be establishing if the cells being used in the experiment can produce and respond to bystander effects in experimental conditions that have been consistently shown to produce bystander effects. These conditions may include the generation of explant cultures (Mothersill et al. 1990, 1995; Belyakov et al. 2003, 2006) and irradiation at set doses with a gamma source. Furthermore, if these additional experiments would not be feasible, perhaps to confirm if the experimental procedure itself can produce RIBE, researchers could use a cell line that has been shown to produce and respond to the bystander effect (e.g. HCT, HaCaT) (Le et al., 2015a, 2015b; Vo et al., 2017b) and/or use *ex vivo* models that have been successfully used in RIBE experiments before, such as murine bladder tissue explants. The use of a secondary tissue or cell line endpoint can be helpful in determining if the effect or lack thereof observed is due to the experimental procedure itself or the kind of cells being used in the experiment. The use of these secondary tissue endpoints can be very useful in neutron RIBE experiments where contradictory findings between different tissue models abound.

4.3. Dose rate and time post irradiation

It is important to note that dose rates were not accounted for in the generation of most of the graphs, excluding Fig. 8 which explicitly shows transformation frequency trends for dose rate. Ideally, each data set would be selected for use in the analysis partly due to it having been generated with consistent dose rate with the rest of the data. Varying dose rates in the data analysis was an inevitability because of the limited data sets that were available in the literature. In the future when more data is collected on neutron RIBE, additional analyses should be performed and compared with the results found in the present study.

It was postulated at the beginning of this study that dose rates may play an important role in whether or not effects are observed in neutron-centric RIBE experiments; different dose rates used by different groups could potentially explain why some researchers observe neutron effects and some do not. While it is difficult to take into consideration dose rates when problems have been herein demonstrated to exist with gamma contamination and when these problems with dose rate may exist, at least in part, due to gamma contamination, an attempt was made to explore the potential factor of dose rate on neutron-based experiments.

The papers describing transformations were used as examples of how different dose rates could potentially lead to different findings. Fig. 8 shows several series of points representing data sets collected after irradiations at a set dose rate. Interestingly, lower dose rate series appear to predominate in the higher transformation frequency region between approximately 100 and 750 mGy, hovering around 1 × 10˚-3. In contrast, series with higher dose rates start lower, around 1 × 10˚-4 at around 100 mGy and appear to increase steadily to 8 × 10˚-4. This effect was also mentioned in all of the papers that were sampled for analysis in this dataset (Listed in Table 3). It was determined through this analysis that dose rates could have an appreciable effect on transformation effects.

The dose rate used for gamma irradiations was also a point of interest because of the potential effects of gamma radiation concurrent with neutron radiation exposure, given that gamma contamination has heretofore been described as an issue with neutron studies. Despite this, a meta-analysis on the gamma radiation data was not conducted. The primary reason for this omission was due to the fact that there exist no papers in the literature from our lab that utilize dose rates low enough to reliably compare to the ones used in Fig. 8. Furthermore, no papers in the literature today describe endpoints for their research that could be compared to the results in Fig. 8.

Due to the observed effects in the transformation datasets, it is not unlikely that dose rates could have an effect on the other endpoints examined in this study. Various other publications have described the effects of varying dose rates on results for other endpoints, albeit in a context unrelated to RIBE.

The papers reviewed unfortunately did not conduct the time-course analyses required to discuss RIBE in terms of the time post-irradiation that the assay was conducted, although this could be a confounding factor between studies. Previous studies on RIBE have noted that time following irradiation influences results collected from several biological endpoints (cite). It is important that such a variable be kept consistent in future studies, wherein the establishment of time-course analyses for the assays being used prior to the experiment proper may be useful.

4.4. Neutron energies

A crucial issue in determining radiation damage on living organisms is the energy spectra of the type of radiation used. This holds true for neutron radiation as well, where authors have reported that particle energy and radiobiological damage are related (Hall et al., 1975; Tanaka et al., 1999). Critically, several research bodies have recommended the use of weighting factors to take energies into account for neutron irradiations; the deposition of energy in a living target are dependent, at least partly, on the energy of the incident spectra. Used for determining the equivalent dose, radiation weighting factors aid in eliciting the relative biological effects when absorbing dose (ICRP, 2007). In the case of photons (i.e. gammas, X-rays), radiation weighting factors are 1, thus, it is safe to assume that the biological effects observed are reflective of the absorbed dose (ICRP, 2007). For neutrons, radiation weighting factors can vary anywhere from 2 to 20 and depending on the energies of the incident neutrons, can drastically affect the outcome of the data observed. Thus, the assumptions that come with photon irradiation cannot be made with neutrons (ICRP, 2007). Among all datasets that were analyzed, the neutron energies consisted of 0.1 MeV, 0.85 MeV, 2 MeV, 2.2 MeV and 5.9 MeV. When utilizing the equations provided in the ICRP 103 (2007), radiation weighting factors were determined to be 10, 21, 17, 17 and 11 for the respective energies. Applying these values to the datasets, we can see the equivalent doses are significantly larger than the absorbed doses. For example, when referring to Table 3 (Miller et al., 1990), with a neutron energy of 5.9 MeV, a 100 mGy neutron dose is equivalent to 1.1 Gy gamma dose. This indicates that although 100 mGy was imparted onto the cells, the cells effectively received 1.1 Gy. This is concerning when considering that the observable biological effects might not be reflective of the physical impact (unlike in the case with gamma irradiation). This is something to account for when performing dosimetry experiments with neutrons on biological samples. Important to keep in mind that radiation weighting factors serve only as a recommendation for better understanding biological effects for varying radiation types.

Accounting for the charged particles that may arise at these neutron energies is also fundamental in providing a physical explanation for the biological phenomena observed. It is known neutrons impart their biological damage through the means of inducing charged particles (i.e. protons and alphas) along with gammas via interactions (Conger and Giles Jr, 1950; Hall et al., 1975; Ritter et al., 1977; Woloschak and Chang-Liu, 1990; Goodhead, 1999). The outcome of these interactions varies with neutron energies, however, mainly comprise of elastic and inelastic scattering along with thermal neutron absorption. With these energies falling within the intermediate and fast neutron ranges, the primary mode of energy transfer is through elastic and inelastic scatter.

Determining the outcome of neutron scattering events becomes increasingly difficult as energies become more alarming, however, chiefly come to down to the type of target nuclei, its respective threshold energy and its angle of scatter. Most commonly, neutrons undergoing elastic scattering will generally give rise to protons of energies up to 1 MeV, depending on target nuclei (Turner). Generally, at these energies, the median neutron energy transfer is considered to be 0.63 times the incident neutron (Turner). This means that if the incident neutron has energy of approximately 1.6 MeV, the ejected proton will be 1 MeV. When considering that for some publication's energies of 2 MeV or greater were stated, it becomes clear that any evidence of a bystander response is being mediated through this particular interaction.; Especially when considering that over 85% of the initial interaction of fast neutrons in soft tissue (consisting of hydrogen, carbon, oxygen, nitrogen), give rise to protons under energies of 10 MeV (Turner) and that proton induced bystander effects have also been documented (Nagasawa and Little, 1992). The frequency of (n,p) reactions can also increase when considering the cross-sections of specific target nuclei. Take for example Chlorine-35, which is naturally abundant and also happens to be found in cells. Considered one of the inorganic ions of the cell, it collectively only accounts for 1% of the cells molecular make-up (Cooper, 2000). Despite its low bioavailability, chlorine serves as a viable source for a variety of neutron-induced secondary particulates. For the relevant neutron energies, chlorine has a (n,p) cross section of 105 mb (millibarns) for a neutron energy of 5.9 MeV (65.2 mb, 58.3 mb, 12.8 mb, 0.025 mb for 2.2 MeV, 2 MeV, 0.85 MeV, and 0.1 MeV respectively) (Kalos and Ray, 1963). At very high energies, such as the case with 5.9 MeV neutrons, the occurrence of alpha particles becomes more apparent. Alpha particles have also

been well documented in inducing bystander effects due to their high LET (Nagasawa and Little, 1992; Nagasawa and Little, 2002). Again, for chlorine, the (n,α) cross section is 150 mb at this energy which, despite it being a relatively larger cross section, its prevalence amongst the reviewed publications is unlikely, considering much of the energies are below 1 MeV. Thermal neutron absorption primarily gives rise to gammas of very defined energies however at increasing energies, the range of gamma energies becomes vast. Simply through fast neutron elastic scatter or inelastic scatter, neutrons can temporarily excite the target nuclei which then give rise to prompt gammas. Considering that these are intermediate and fast neutrons, they would require a high stopping power before reaching thermal levels, providing opportunity for the emission of numerous gammas. With cross-sections of 0.035 mb and 1.243 mb for chlorine at energies of 0.85 MeV and 0.1 MeV respectively (negligible at the other energies), there is no shortage of secondary gammas from neutron scattering events. Once reaching thermal levels, neutrons can be completely absorbed due to the presence of hydrogen, which with a cross-section of 0.33 b, gives rise to 2.2 MeV gammas (Turner). From the current literature, it is clear that gammas induce bystander effects and when considering the number of secondary emissions, especially with energies up to 2.2 MeV, it is clearly evident that the (n,γ) interactions are the primary facilitators of the RIBE at low to intermediate neutron energies. Not only is there contamination in the incident neutron radiation but also in the biological sample, as neutrons give rise to various other forms of radiation. The likelihood of these secondary radiations are outlined above, however, only for one specific target nuclei. When considering all other potential target nuclei present in the cell (i.e. hydrogen with a 70% abundance), it becomes extremely difficult to interpret the complex radiation field that is induced by neutrons (with primary focus on gammas for this review). To do so it would require simulations (i.e. Monte Carlo) to evaluate the various track damages which cannot be achieved experimentally. However, it is still concerning that the studies reviewed in our analyses make no considerable attempt to mitigate the contributions from a contaminated neutron beam and also do not take into account the equivalent doses that could aid in better understanding the biological ramifications of neutron irradiation.

4.5. Evidence against neutron RIBE

Several papers that were reviewed have found that treatment with neutron radiation does not produce RIBE for certain endpoints in certain cell lines. A paper by Liu et al. (2006) found that treatment with neutrons up to 1 Gy did not produce a reduction in clonogenic survival in reporter cells. A dose threshold for gamma effects was established and accounted for in the neutron irradiations. The doses for the experiments were 0.7 mGy/min for the lower doses and 25 mGy/min for the higher “preliminary” dose. Another paper using human lymphoblastoid cells also did not find evidence of RIBE (Seth et al., 2014). The authors used micronuclei formation as their primary endpoint for RIBE, which has been used by other publications in the literature previously (Baskar, 2010; Reisz et al., 2014; Havaki et al., 2015; Le, Fiona E. McNeill et al., 2015a, 2015b). Gamma effects were accounted for in this paper through a separate experiment and the researchers used doses ranging from 0.5 to 4.0 Gy. Another paper using an *in vivo* irradiation model with zebrafish followed by a reporter assay using HPV-G found no effects of neutron irradiation (Wang et al., 2011). This paper accounted for gamma contamination, however there was a lack of effect even when the gamma dose was above the threshold. This is evidence, as described previously, that tissue from the caudal fin does not seem to have the capacity to generate bystander signals after exposure to neutrons.

4.6. Potential molecular explanation

Many papers in the literature have described RIBE attributable to particle-beam radiation. In fact, one of the earliest papers to describe this RIBE used alpha particle radiation. As reviewed, there is evidence that RIBE are the result of the activation of multiple signalling pathways that culminate in the release of soluble factors into intercellular space, followed by signal transduction in recipient cells (Nagasawa et al., 2002; Azzam et al., 2003; Zhou et al., 2005; Hei, 2006; Prise and O'sullivan 2009).

The finding that lower dose rates seem to produce a more pronounced effect in neutron-based studies may provide some evidence as to what molecular mechanisms contribute to RIBE (Fig. 8). If, conversely, higher dose rates produced a more pronounced effect, it is likely that structural damage to cells could serve as the primary impetus to the emission of a bystander signal (Dilmanian et al., 2007; Wideł et al., 2009). Because this is not the case according to our analysis, evaluation of various established molecular RIBE signalling pathways must be completed, in order to elucidate potential emerging permutations in the numerous signalling cascades involved in RIBE.

Alpha particles being a form of high LET radiation seem to exhibit similar properties as neutrons when interacting with tissue. Surrounding research could exemplify low dose tissue interactions for high LET radiation, specifically for neutrons. Experiments conducted by Nagasawa and Little (1992) found pronounced effects due to alpha particles in the 0–5 mGy range. Alpha particles appear to indirectly induce cell death and mutations more efficiently in mammalian cells than EM radiation (Nagasawa and Little, 1999). Because the majority of studies analyzed in the present study did not take into consideration the potential effects of gamma contamination, it is unclear if neutron radiation alone contributes predominantly to these effects at low doses. However, at higher doses, the effect of neutron radiation appears to predominate according to our meta-analyses. The question of whether or not neutrons produce the same effects as alpha particles can help elucidate what mechanisms are at play in particle-based RIBE. Further research must be done in order to validate the dose response curves generated by our analyses before any comprehensive hypotheses can be proposed.

5. Conclusion and future directions

The supporting literature surrounding RIBE and associated NTE has grown substantially ever since its introduction in 1954 and it has strengthened our understanding of bystander effects when cells interact with electromagnetic radiation. However, despite being an extensive area of research, a sizable gap in our understanding of neutron related effects seems to still be present. Confounding factors like high gamma contamination, different cell lines, and dose rates skewed our ability to formulate an accurate relationship between neutron irradiation and RIBE. The effects of neutrons in low dose regions still needs to be explored. Nonetheless, after performing the meta-analysis we have determined that neutrons could potentially induce RIBE at higher doses, while gamma effects still seem predominant at lower dose regions. For future experiments, a comprehensive study of gamma dose thresholds for various cell lines should be established in order to further elucidate neutron induced RIBE. Variables such as dose rates, tissue modelling choice, and energies should also be kept consistent within these studies to limit the number of potential variables that could make results challenging to interpret and discrimination of potential neutron effects difficult if not impossible. Other factors, such as time following irradiation that the assay is conducted, are also likely important in determining consistency between studies and groups. The shortcoming in the literature seeking to outline the indirect effects of neutron radiation is overall a problem because it affects our ability to potentially distinguish the molecular mechanisms underlying effects from different sources of radiation. This could have vast implications in our present understanding of NTE and RIBE. Effects such as gap-junction intracellular communication, calcium signaling, and biophoton signaling still need exploration as RIBE of neuron radiation to the extent that they

have been explored as indirect effects of gamma and alpha radiation. Careful understanding of the indirect effects of neutron radiation has far-reaching implications not only to human health but also to the protection of ecosystems and an environment suitable for life in general. Because the effects of neutron radiation on living systems are relevant to both organisms and the environments they inhabit, elucidation of RIBE and other indirect effects is crucial for risk assessment. It is our hope that these recommendations will assist in upcoming research that will inform policies on radiation protection in the future.

- A sizable gap in our understanding of neutron-related indirect effects exists.
- Confounding factors make interpreting the present data and correlating it with other studies difficult:
  - High gamma contamination.
  - Different cell lines.
  - Dose rates.
  - Energies.
- The effects of neutrons in low dose regions still needs to be explored.
- Gamma effects seem predominant at lower dose regions.
- Overall, a lack of robust conclusions affects our ability to potentially distinguish the molecular mechanisms underlying effects from different sources of radiation and has implications in our understanding of NTE and RIBE.
- Elucidation of RIBE and other indirect effects is crucial for risk assessment for humans, animals, and the environment.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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