Peer Review File

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Reviewer A

In his manuscript „Morphofunctional characteristics of proliferation and apoptosis of spermatogenic epithelium: pathogenesis of temporary azoospermia after local electron irradiation", Demyashkin studies the damage of the seminiferous epithelium caused by a single dose of local electron irradiation. His aim is to characterize the damage in detail to shed light on the pathogenesis of the temporary azoospermia that occurs after local irradiation. To achieve this goal, he takes a close look at a set of cellular markers of proliferative and apoptotic pathways, in detail: expression of Ki-67, caspase 3, p53, bcl2 and TUNEL staining.

The study reveals a time-dependent damage to a part of the seminiferous tubules, which is accompagnied by an increase in apoptotic markers and decrease in proliferative and anti-apoptotic markers.

Although this outcome is quite predictable, it might be that this special constellation (single injection, dose of 2Gy, rat model, time course analysis) has never been investigated in detail, however, if this is the case, the author should make it clear in the discussion. What distinguishes this study from others?
Reply: We indicated it in the highlight box

What has already been published that is comparable? How do the presented data fit into the picture? What is different compared with literature?

Reply: We have indicated data from the specialized literature in the text of the Discussion section (Lines 247-261)

Comments on the Material and Methods part:

The author writes that animals were withdrawn gradually: 1 week after irradiation, and then every two weeks. This would mean the following timepoints: 1week, 3weeks, 5weeks, 7weeks, 9weeks, 11weeks. However, this does not correspond to the presented timepoints.

Reply: We've updated the timelines

Also, many of the timepoints are missing in the Results part of the paper. If animals were observed at all of these timepoints, where are the data? How many animals were observed at each single timepoint?
Reply: We have updated the timelines and data in the Results section (Fig.1, Tables 1,2; lines 128, 130, 134, etc)

Throughout the whole manuscript the description of timepoints varies considerably: The author switches between month, week and even days (Table). This is very annoying. Please use only one scale and stick to it.

Reply: We have indicated timelines throughout the text in weeks

The practice of counting cells in 10 randomly selected fields of view is problematic when only a part of the tubules is damaged. This can lead to big deviations from the real situation dependant on the

selection of the field. I suggest to rather quantify the damage per tubule and select the affected ones, assuming that the not affected tubules are completely unaffected. This could be quantified beforehand, as the author has done.

Reply: We have clarified this in the Methods section (lines 95-97)

A good measure of sperm production and irradiation damage would be the sperm number. Why has this not been assessed at all?

Reply: This is due to the fact that there were no gametes in the lumen of the seminiferous tubules.

Comments on the Results:

Fig.1: The author writes that the damage occurs in 1/8 of the tubular area: how has that been measured? By counting the tubules or by real area measurements? Which time point corresponds to this result? After 2 months the damage corresponds to 1/4 and later to 1/3 of the area. Does the progrediency cease at some point? Will there be a full restoration of the damage? When will that be?

Reply: Data on missed dates added. By the end of the experiment, a complete recovery of spermatogenesis was not observed, however, we found a trend towards its recovery (appearance of spermatogonia and their proliferation at the final stages of the experiment).

The presented H&E pictures are in my view not comprehensive: I suggest to show first overview pictures at all stages, so that the reader can see the gradual aggravation.

Reply: Data on missed dates added.

The damaged tight junction and blood testis barrier is not a result, as it has not been tested. If the author wants this to be part of the Results section, it has to be tested, otherwise it is speculation and belongs to the discussion part.

Reply: This has been moved to the Discussion section.

The detailed pictures in Fig.1 should be labeled much better, you can use asterisks, arrows etc. so that the reader can find the described features.

Reply: We have labeled it better.

Also the term “seed balls” is not a very scintific term. I assume you mean the multinucleated giant cells.

Reply: We have corrected this

Line 111: the author describes a restoration of the number of germ cells, mainly due to spermatogonia and spermatocytes. How is this number achieved? Did the author count the number? How high is the number? How was the counting done? Did the author discriminate between spermatogonia and spermatocytes? And if yes, how? Which markers were used? As the group of germ cells consists of spermatogonia, spermatocytes, spermatids and mature sperms, the statement, that restoration was due to increased numbers of spermatogonia and spermatocytes is needless, as spermatids and sperms develop from them, so it is pretty clear, that the earlier stages have to increase before the later stages can increase. Also here a diagram with numbers would be helpful.

Reply: We have clarified this in the Results section (highlighted in yellow)

Line 118: the number of Ki-67 positive Leydig cells increased. Did the number of Leydig cells increase? Or only the number of Ki67-positive Leydig cells? Was this differential calculation done? Can the author show labeled pictures to proof this?

Reply: This is declared in the Results (line 154)

Table 1 and Fig.2:

How should the table be read? The numbers are % of what? The control has 76% Ki67-positive cells of all counted cells? If yes, which cells were counted? Only spermatogonia and spermatocytes? Or also round spermatids and even sperms? At which time in cell cycle do these markers increase? Which cells in detail are affected?

Reply: Round spermatids and even sperms were practically absent. This was observed at all stages of spermatogenesis only in spermatogonia and spermatocytes, since it was already written that no other cells of the spermatogenic epithelium were observed (only single spermatids were stained).

How do the numbers presented in the text (e.g. line 117 “2,5 times”) fit to the table? How was that calculated? What means “2,5 times in the first month”, when data presented in the table are from day 14?

Reply: We have corrected this

These questions apply to all the results presented in the table. The table should also show all the time points. I would prefer to read diagrams and see more representative pictures than the few ones presented in Fig2. to draw my own conclusion.

Reply: The table also show all the time points

Line 131: “2,5 times larger” Are Leydig cells really larger? How was that measured? Or did the number increase?

Reply: We have clarified it (Line 143-144)

Fig.3: Which cells are TUNEL positive? Which cells were counted, only meiotic cells, or all cells including sperms? If the latter is the case, than 16% off all cells in healthy seminiferous epithelium seems a quite high number. The number increases 1,4 times after irradiation. Does it mean that the TUNEL positive cells are now 22,4% of all cells and at the end of the experiment 75%? If yes, the is not reflected in the picture. Also here I would prefer to see representative pictures of the time course, especially if the effect is so dramatic.

Reply: In order for our figures to be the most informative, we added microphotographs of the TUNEL method at all the studied periods.

Fig. 3 : The word “glow” is not very scientific

Reply: We have corrected this

Comment on the discussion:

The discussion is quite comprehensive regarding the possible (and known) effects of irradiation damage in the testis. However the presented results should be discussed in light of the literature. What is really new? What is maybe contradictory? Also some words on reversibility are missing. Is there (or will there be) a full restoration of the damage? How long after irradiation would that be?

Reply: We have added this to the Discussion section (Lines 256-261).

In conclusion, I think the study is lacking major scientific and methodological issues and therefore I would not recommend the publication.

Reviewer B

This study investigated the pathogenesis of temporary azoospermia after local electron irradiation and identified several key factors involved in the process. The study is well-documented and conducted. I have a couple of questions:

1) Why did the author choose a dose of 2 Gy?

Reply: We have clarified it (Line 193-197)

2) In the conclusion, the authors state a tendency of recovery in the 3rd month, can you please extrapolate this and what it would imply for longer treatments?

Reply: We have clarified it (Line 193-197, 256-261)

3) Could you reformulate the last paragraph of the conclusion and include the pathways involved?

Reply: The pathways have been detailed in the Discussion and cannot be described in the Conclusion.