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Heat induced differential pattern of DNA fragmentation in male germ cells of rats

Kuldeep Kaushik[a,b,*], Naveen Kaushal[c], Pawan Kumar Mittal[a], Natwar Raj Kalla[c]

[a] Department of Zoology, Panjab University, Chandigarh, 160014, India
[b] Dev Samaj College for Women, Ferozepur City, Punjab, 152002, India
[c] Department of Biophysics, Panjab University, Chandigarh, 160014, India

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ABSTRACT

Spermatogenesis being a highly dynamic processes is highly vulnerable to various stresses including heat stress. Though, the relationship between physiological temperature and male germ cells is certainly immense, the magnitude of spermatozoal damage after exposure to heat is evidently degree and dose dependent. Further, there are contradictory reports related to germ cells apoptosis in relation to temperatures. Thus, currently the dynamics of temperature and time dependence on germ cell apoptosis were studied by modulating the heat treatment strategies. It was observed that the rate of apoptosis increased initially then decreased with time. The DNA fragmentation in the 10,000 × *g* supernatant of testis homogenate of rats that received heat treatment for 15-min, 30-min as well as 45-min treatment with 15-min intermittent period was found to be almost equal. In various heat treated animals, the apoptosis was found to be maximum after day-1 of treatments, which then followed a decreased pattern. These results indicate that there may be an initial induction of apoptosis in the germ cells, which later primed or programmed the other germ cells to activate protective mechanisms against heat induced DNA damage and thus protecting germ cell population to undergo apoptosis at later durations.

1. Introduction

A unique feature of the male gonads of many species is their location outside the main body cavity. This feature enables the testicular cells to have a significantly lower growth temperature relative to cells of other tissue. It is well known that the scrotal testes of mammals maintain an appreciably cooler environment than the body. The scrotal temperature in most of the mammalian species is 2-8 °C lower than the abdominal temperature (Harrison and Weiner, 1949; Halder et al., 2018). Spermatogenesis being a dynamic process involving various stages of spermatogenic cycle i.e., meiotic division and spermiogenesis, is relatively most vulnerable to various stresses including heat stress (Lue et al., 2002; Zhou et al., 2002).

It has been reported that slight changes in the physiological or environmental temperatures can induce various signaling pathway that can modulate the vitality of germ cells and/or trigger the death response. Higher environmental temperatures not only reduce the sperm production but also promote the cell death in germ cells as well as Leydig cell (Costa et al., 2018). In buffalo bull increased scrotal surface by heat stress has been correlated with the reduced semen quality (Ahirwar et al., 2018).

Scientific data also revealed that the process of male germ cell death is not a random process but take place in an organized way in various specific stages of the spermatogenic cycle under heat stress (Lue et al., 1999). Temperature sensitivity in male germ cells could be due to a lower relative thermal stability of germ cell-specific proteins (Wolgemuth and Watrin, 1991) or may be due to specific sensor or thermo receptor. Okazawa et al. (2013) also reported that DNA-dependent protein kinase inhibition might reinforce hyperthermia-induced cell death. Heat shock triggers apoptosis in male germ cell *via* both extrinsic and intrinsic pathways where activities of Caspase-8 and Caspase-9 are significantly enhanced (Wang et al., 2012).

Though, the relationship between normal growth temperature and the body temperature in male germ cells is certainly immense, the magnitude of spermatozoal damage after exposure to heat is evidently degree and dose (period of heat exposure). There are contradictory reports related to germ cells apoptosis in relation to temperatures. For example, some reports suggest that apoptotic germ cells are first observed 8 h after exposure at 43 °C for 20 min. In contrast, a short exposure of the testes to lower temperatures of 39°–40 °C has no obvious effect on germ cell death (Rockett et al., 2001). Previously it was also reported that male germ cells seem to have a threshold of

* Corresponding author. Department of Zoology, Dev Samaj College for Women, Ferozepur City, Punjab, 152002, India.
E-mail address: kaushik41738@yahoo.co.in (K. Kaushik).

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time–temperature dosage for apoptosis (Steinberger and Dixon, 1959). Further, Kim et al. (2013), suggested the requirement of a time–-temperature dosage for germ cell apoptosis. Therefore, the aim of this research is to provide a clear information not only of the effect of heat stress on male germ cells apoptosis, but also point to the thresholds of such stress enabling the understanding for further investigations. The present study investigates and establishes relationship between specific heat stress and death of germ cells.

2. Materials and methods

2.1. Animals

Male albino rats (Wistar strain) having body weights in the range of 260–290 g, were procured from the Central Animal House, Panjab University, Chandigarh. The animals were housed in polypropylene cages bedded with sterilized rice husk in a temperature regulated environment of 21 ± 1 °C with 12 h of dark and light cycle. The animals were provided with standard animal pellet diet (Ashirwad Industries, Tirpari, Kharar, Distt. Ropar, Punjab) and water *ad-libitum.* This work was approved by the Research Degree Committee of Panjab University, Chandigarh, Union Territory, India (case no. 14870/Ph.D). The experimental protocols were performed in the Dept. of Zoology, Panjab University, Chandigarh and the care and treatment of animals were also in accordance with ethical guidelines issued by Panjab University, Chandigarh Before commencement of various treatments, the animals were kept for acclimatization to experimental conditions for one week and the final day of their acclimatization period was considered as day zero of the experiment.

2.2. Experimental design

Following the acclimatization animals were randomly divided into two major groups namely *Heat-treated animals* (heat stress) and *Control animals*. The animals were then segregated into subgroups and were processed for investigations as outlined below:

Heat-treated animal groups were divided into three subgroups with atleast n = 6 in each subgroup according to following treatment.

- 15-min heat-treatment
- 30-min heat-treatment
- 45-min treatment with 15-min intermittent period

The effects of heat-treatment were studied after day 1, 2, 4 and 7.

2.3. Induction of testicular hyperthermia

First of all, the rats were anesthetized with an intra-peritoneal (IP) injection of sodium pentobarbital (40 mg/kg body weight). After anesthesia, the rats were shifted to hyperthermic treatment apparatus. The rats were placed in thermostatically controlled water-bath at 43 °C in such a way that their scrotum containing testes gets immersed in water for homogeneous hyperthermic exposure as suggested by previous investigations (Lue et al., 1999, 2002; Zhou et al., 2002; Wang et al., 2012). The hyperthermic exposure was employed over different groups of animals for various time periods as mentioned earlier. After heat treatment, animals were released from the said apparatus and allowed to recover from the stress at room temperature before being returned to their respective cages.

2.4. Collection/preparation of samples

After the different treatments were over, the animals were sacrificed under light ether anesthesia after different time intervals as mentioned earlier. The testes were extracted out, weighed carefully and were processed for assessment of apoptosis. Male germ cell is an excellent model of rapid apoptotic induction system, which shows early ladder with late smearing pattern of DNA. In the present investigation, assessment of apoptosis was carried out by *spectrophotometer estimation of DNA content in* 10, 000 × *g supernatant and* DNA fragmentation assays.

2.5. Assessment of apoptosis

2.5.1. DNA isolation

The DNA was isolated from fresh or stored samples by organic method proposed by Adeli and Ogboma (1990) with slight modifications. A 10% testicular homogenate was prepared in lysis buffer (10 mM Tris-Cl, 0.1 M EDTA, 0.5% SDS) and put it water bath at 58 °C over night. To the suspension proteinase K (20 mg/ml) was added followed by addition of an equal volume of PCA (phenol buffered with Tris-HCl (pH 8.0), chloroform and isoamyl alcohol in the ratio 25: 24: 1) and mixed for 15-min to form an emulsion and then centrifuged at 2500 rpm for 15-min. The supernatant was collected and above mentioned step was repeated once more with chloroform and isoamyl alcohol (CA) in the ratio 24:1. To the supernatants 1/10 volume of 3 M sodium acetate solution (pH 5.2) was added and mixed, followed by addition of chilled ethanol to precipitate the DNA. The DNA was washed with 70% ethanol, air dried, suspended in 10 mM Tris-EDTA buffer (pH 8.0) and stored at 4 °C till further use.

2.5.2. Spectrophotometer estimation of DNA content

For estimation of DNA in 10, 000 × *g* supernatants, aliquots of the latter were treated with 2.5% TCA. After precipitation of the proteins, the mixtures were centrifuged at 3000 × *g* for 10 min. Aliquots of the supernatants thus obtained were added to the TE buffer (1X) for reading of absorbance at 260 nm using UV-VIS spectrophotometer (Hitachi make). Samples containing the known amount of Whale sperm DNA were also read at said wave lengths for the preparation of standard-curve in order to calculate the DNA content in the test samples.

2.5.3. DNA fragmentation analysis

Electrophoresis of DNA was carried out for DNA fragmentation analysis on 2.5% agarose gel (w/v) prepared in Tris-Borate-EDTA (TBE) buffer (pH 8.0). About 8 µg of DNA sample was mixed with loading buffer (10% glycerol and 0.025% bromophenol blue in water) and subjected to electrophoresis. DNA bands stained with ethidium bromide were visualized on a UV trans-illuminator and photographed with the help of gel documentation system (Ultra CAM make) for further analysis of electrophoretic patterns obtained.

2.6. Statistical analysis

Statistical analysis was carried out employing Statistical Package for the Social Sciences (SPSS, version 10.00, SPSS Inc, Chicago, Illinois, USA) software. Data were expressed as Mean ± SD for observation in each group. The statistical significance of inter group difference of various parameters were determined by unpaired student's t-test. Comparison was made between the test groups to that of the control group of rats and the statistical significance was depicted employing symbol "*".

3. Results

3.1. DNA fragmentation studies

Gel documents of agarose gel electrophoresis depicted the fragmentation pattern in all treatment groups. All the lanes except marker (ladder-100bp) contained equal amount of DNA. Electrophoresis of 10,000 g supernatant depicted fragmentation (characteristic of apoptosis) that was in line with duration and temperature. Fragments of 180bp and 360bp were clearly visible in samples from all treatment groups after day 1. The amount of 180bp and 360bp DNA gradually

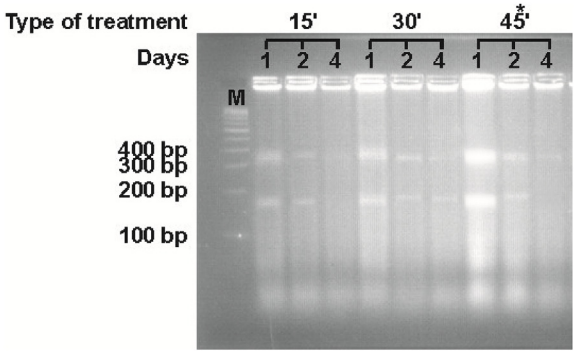

Fig. 1. Electrophoresis of 10, 000 × *g* supernatant of male germ cells of various heat treated animals groups in 2.5% agarose gel. M - Marker, a hundred base pair ladder. 45 *- 45-min treatment with 15-min intermittent periods.

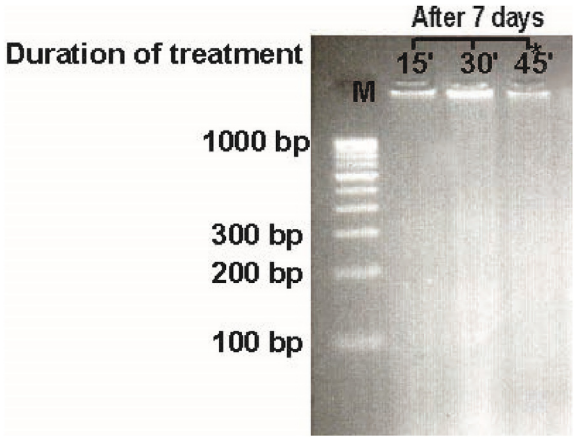

Fig. 2. Electrophoresis of 10, 000 × *g* supernatant of testicular homogenate in 2.5% agarose gel. M - Marker, a hundred base pair ladder. 45* - 45-min treatment with 15-min intermittent periods.

decreased after day-2 and day-4 (Fig. 1). Moreover, the intensity of 360bp band appeared to be comparatively higher than band of 180bp in almost all treated groups. The characteristic bands of apoptosis did not appear in any group after day-7 from the treatment (Fig. 2).

In Fig. 3, the band of Low molecular weight (LMW) DNA was not visible in the electrophoresis of genomic DNA of various treated group. Severe high molecular weight (HMW) DNA fragmentation or smearing effects are visible in various groups of animals after day-2. Maximum fragmentations were observed in the HMW DNA of male germ cells that received heat treatment for 30-min as well as 45-min treatment with 15-min intermittent periods after day-2. Severe HMW DNA fragmentation was seen in the animals that received heat treatment for in 30-

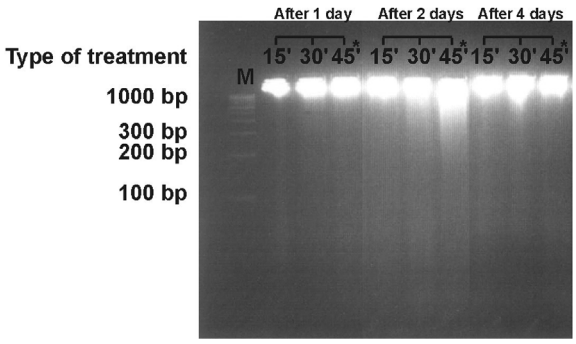

Fig. 3. Electrophoresis of the DNA samples obtained from the tissue homogenate of male germ cells in 2.5% agarose gel. M - marker, a hundred base pair ladder. 45* 45-min treatment with 15-min intermittent periods.

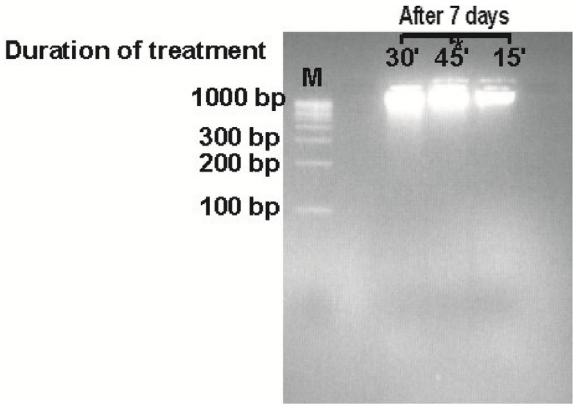

Fig. 4. Electrophoresis of the DNA samples obtained from the tissue homogenate of male germ cells in 2.5% agarose gel. M - marker, a hundred base pair ladder. 45*- 45-min treatment with 15-min intermittent periods.

min as well as 45-min with 15-min intermittent period after day-7 (Fig. 4).

3.2. LMW DNA content in 10,000 × *g* supernatant of heat treated animals

Day-1 after heat treatment at 43 °C for different time duration studied, the content of LMW DNA in the 10,000 × *g* supernatant of testicular homogenate increased when compared to that of control animals. Maximum content of LMW DNA was observed in supernatant of testicular homogenate of animals that received 45-min heat treatment with 15-min intermittent periods. The animals that received heat treatment for 45-min with 15-min intermittent periods have ~30% higher values of LMW DNA from 15-min heat-treated animals group. The values of 15-min and 30-min heat-treated animals were found almost equal (Fig. 5).

After day-2 the LMW DNA content in the supernatant of testicular

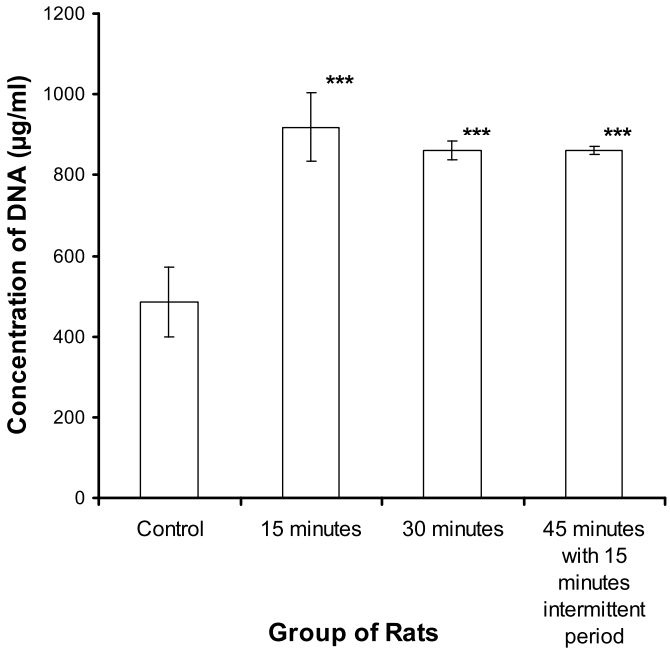

Fig. 5. LMW DNA content after day-1 in 10,000 g supernatant of testicular cells treated at 43 °C of various animal groups. Data is represented as mean ± SD from 6 observations. Symbols and statistical significance: * and *** represent $p \leq 0.05$ and $p \leq 0.001$ respectively when various heat -treated animals compared to control counterparts. Mean value are in µg/ml of 10,000 g supernatants.

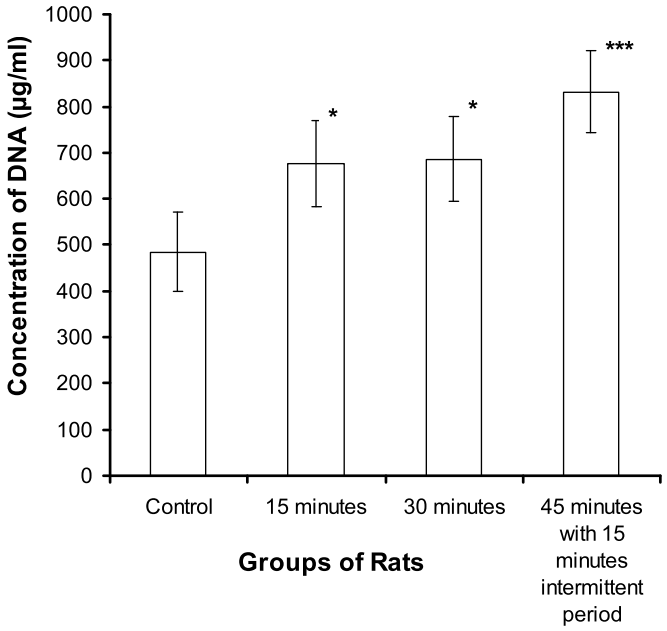

Fig. 6. LMW DNA content after day-2 in 10,000 g supernatant of testicular cells treated at 43 °C of various animal groups. Data is represented as mean ± SD from 6 observations. Symbols and statistical significance: *** represent $p \leq 0.001$ when various heat -treated animals compared to control counterparts. Mean value are in µg/ml of 10,000 g supernatants.

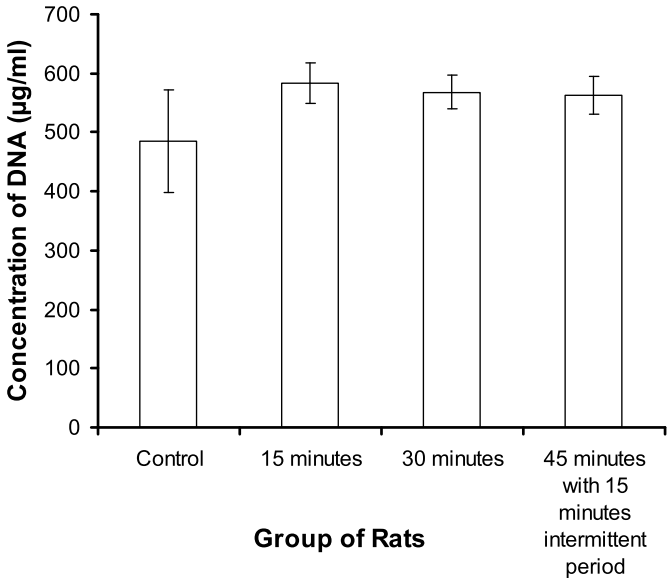

Fig. 7. LMW DNA content after day-4 in 10,000 g supernatant of testicular cells treated at 43 °C of various animal groups. Data is represented as mean ± SD from 6 observations. Symbols and statistical significance: * and *** represent $p \leq 0.05$ and $p \leq 0.001$ respectively when various heat -treated animals compared to control counterparts. Mean value are in µg/ml of 10,000 g supernatants.

homogenate increased significantly in all heat-treated animal groups as compared to control counterpart. Maximum LMW DNA content was observed in animals that received the heat treatment for 15-min. The amount of LMW DNA in 15-min heat treatment animals group was slightly greater than other two groups, which was non-significant whereas the amount of LMW DNA in another two groups was almost the same (Fig. 6).

After day-4, the content of LMW DNA in 10,000 g supernatant of testicular homogenate was found to be increased when all heat treated animal groups compared to control counterpart. LMW DNA in 45-min with 15-min intermittent periods treated animals was also statistically significantly lower than the animal that received the treatment for 15-min whereas in 30-min heat treated animals, it was greater than 15-min heat treated animals group (Fig. 7).

After day-7 of heat treatment at 43 °C for different time duration studied, the content of LMW DNA in 10,000 g supernatant of testicular homogenate was found to be slightly increase when all compared to control counterpart. There is not any statistical relationship among the concentration of LMW DNA in the testes of various groups of treated animals (Fig. 8).

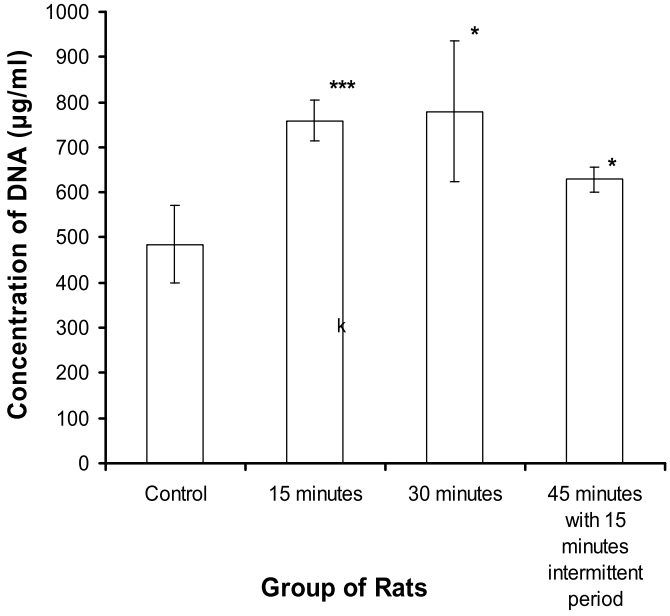

Fig. 8. LMW DNA content after day-7 in 10,000 g supernatant of testicular cells treated at 43 °C of various animal groups, Data is represented as mean ± SD from 6 observations. Mean value are in µg/ml of 10,000 g supernatants.

4. Discussion

Theme of male contraception has largely based on the approaches that induce azoospermia or severe oligospermia through accelerated germ cell/apoptosis induced by physiological and biological factors. Thus, understanding the specific steps in the germ cell apoptotic pathways that are affected by male contraceptives will allow more specific targeting in future male contraceptive development. In this regard, heat sensitive nature of spermatogenesis has been one of the areas of research interest for scientists worldwide. Sinha-Hikim et al. (2003), suggested that instant induction of apoptosis in male germ cells after a single heat exposure is excellent *in vivo* model system for studying the various signaling pathways and mechanisms of programmed germ cell death.

Germ cells containing significant amount of genetic material have an average length of 27 kb and its repair and maintenance goes through highly specific processes. Since the DNA wrapped around the histones comprises ~180-200bp, appearance of this multiple interval in the form of ladder or apoptotic ladder occur by the activation of endogenous endonuclease that cleave the DNA in linker regions between histones on the chromosomes. Since in some model systems, cell viability does not diminish until 4–6 h after treatment (Bortner et al., 1995), these types of DNA degradation clearly occur before cell death. Once these cells have fragmented their DNA they are committed to die and cannot be rescued by removal of the apoptotic signal.

Data of DNA fragmentation from agarose gel electrophoresis after day-1, day-2, day-4 and day-7 of various heat-treated animals shows

that the intensity of fragment of 180bp and 360bp were dominant in all treated groups after day-1 and then decreased with time. This might be due to heat stress induced pre-apoptotic condition making various developmental stages of spermatogenesis to undergo death or activate repair mechanisms culminating in either formation of viable sperm or degrade themselves. It may be postulated that due to lack of sufficient cytoplasm majority of germ cells are highly sensitive and slightly change in the internal milieu lead to cell death after receiving heat treatment in various groups of animals. Due to instant induction of apoptosis in male germ cells the intensity of DNA fragmentation was found to be higher after day-1 of treatment. Ohta et al. (1996) also reported DNA fragmentation increased at day-1 post cryptorchid day followed by a gradual decrease thereafter. Reports suggest that the number of apoptotic cells in the cryptorchid testes showed a 7-fold increase at 1-day post cryptorchid as compared to the normal counterparts then it gradually decreases (Ohta et al., 1996). Lue et al. (1999) also observed LMW DNA fragmentation to be evident within day-1 and day-2 after heat treatment and that almost disappeared by day-9 after heat exposure.

Electrophoresis of genomic DNA after day-2, the group of 30 as well as 45-min treatment with 15-min intermittent periods showed smearing effect, which is indicative of rapid apoptosis showing early ladder and late smearing effects. Earlier studies have also shown that the early morphological change of nuclear chromatin coincides with the appearance of HMW fragments, whilst the formation of the DNA ladder (DNA fragmentation) is a rather late event, occurring during or after apoptotic body formation has taken place (Walker et al., 1994; Weis et al., 1995; Cohen et al., 1994). Collins et al. (1997) however, suggested that rapid apoptotic induction system shows early ladder with late smearing pattern of DNA in agarose gel, which might be a consequence of effects of heat induced oxidative stress on male germ cell (early laddering and late smearing affect of male germ cells). Severe HMW DNA fragmentation was also seen after day-7 in 30-min as well as 45-min treated animals with 15-min intermittent period of heat stress. This was due to the effect of heat induced oxidative stress on male germ cells (data communicated). Barroso et al. (2000), found a positive and significant correlation between ROS and DNA fragmentation. ROS mediated high frequencies of single and double-stand DNA breaks have been observed in the spermatozoa of infertile male (Aitken and Krausz, 2001). Furthermore, studies suggest that exposure of sperms to artificial produced ROS resulted in a significant increase in a DNA damage in the form of modification of all base, production of base free sites, deletion, frameshift, DNA cross links and chromosomal rearrangements (Twigg et al., 1998; Duru et al., 2000).

These DNA damage induced fragments can readily be demonstrated by agarose gel electrophoresis as DNA ladder which is a clear indication of apoptosis. Concentrations of LMW DNA in various heats treated animals, directly indicates increased rate of apoptosis with increase in the duration of heat treatment up to an optimum time. After day-1, animals that received 45-min treatment with 15-min intermittent periods, the concentration of LMW DNA was maximum, which directly correlated to apoptosis in male germ cells. After day-2 from the treatment, maximum concentration was found in the animals that received heat treatment for 15-min. Various groups of animals that were sacrificed after day-4 from the treatment, maximum amount of LMW DNA was found in the 10,000 g supernatant of animals that received heat treatment for 30-min. The average of fragmented DNA in the 10000 g supernatant of testis homogenate of rats that received heat treatment for various times (15, 30, 45 min) was found to be almost equal. It may be suggested that instead of initiation of apoptosis, heat induces DNA fragmentation or apoptosis in the initial stage of physiological response in the male germ cells and have similar effect on cell death. The extent of apoptosis was found to be almost equal in all three heat treatment protocols. In agreement with current results, previous studies from our lab also suggested that male germ cells have very short duration to elicit and complete the process of apoptosis (Kaushik et al., 2018).

5. Conclusions

It is concluded that due to involvement of rapid apoptotic system induced by heat treatments, there is an initial substantial increase in the rate of apoptosis, which decreased with the time. This may be due to decrease the population of apoptotic potent germ cell with times. However, the extent of apoptosis was found to be almost equal in all three heat treatment protocols. It may be suggested that instead of initiation, heat induce DNA fragmentation or apoptosis in male germ cells in the initial. In various heat treated animals, the apoptosis was found maximum in the sample of animals that were sacrificed after day-1 and the same then decreased with time.

Author contribution

KK conceived the study, participated in its design, analyzed the available data and helped to draft and edit the manuscript. NK helped to design, draft and edit the manuscript. PKM and NRK supervised the research work, revised it critically for important intellectual content; and given the final approval of the version to be published. All authors read and approved the final manuscript.

Declaration of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as real or potential conflict of interest.

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2019.07.021.

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