The Egyptian Journal of Medical Human Genetics (2016) 17, 173–184

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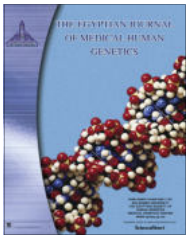

ORIGINAL ARTICLE

Inducible protective processes in animal systems XV: Hyperthermia enhances the Ethyl methanesulfonate induced adaptive response in meiotic cells of grasshopper *Poecilocerus pictus*

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Received 10 October 2015; accepted 16 November 2015
Available online 7 April 2016

KEYWORDS

Adaptive response;
Hyperthermia;
Ethyl methanesulfonate;
Poecilocerus pictus

Abstract *Purpose:* To understand the role of hyperthermia in adaptive response, Ethyl methanesulfonate (EMS) an anticarcinogenic agent, adapted meiotic cells of *Poecilocerus pictus* was used.

Materials and methods: Based on the pilot toxicity study, the effective higher temperatures of 40 °C and 45 °C for 15 or 30 min were chosen. *P. pictus* were treated with conditioning (L) or challenging (H) doses of EMS and 2 h time lag (TL) between these doses (L-2 h-H) was employed. Different treatment schedules were used to analyze the influence of hyperthermia on EMS induced adaptive response namely (i) pre treatment; (ii) inter treatment; (iii) post treatment and (iv) cross adaptation. After each treatment schedule, animals were sacrificed at 12, 24, 36 and 48 h recovery times, testes were processed for meiotic chromosome preparations and anomalies were analyzed.

Results: The frequencies of anomalies induced by both conditioning and challenging doses of EMS were significantly higher ($p < 0.05$) compared to those of the control and hyperthermia groups. The combined treatments resulted in 44–50% reduction compared to additive effect of EMS. The pre, inter, post and cross adaptation treatments with hyperthermia significantly reduced the frequencies of chromosomal anomalies compared to the challenge and combined treatments with EMS at all recovery times ($p < 0.05$) tested.

Conclusion: There is a protection against EMS induced anomalies by hyperthermia in *in vivo P. pictus*. As far as our knowledge is concerned, this is the first report to demonstrate that hyperthermia enhances the EMS induced adaptive response in *in vivo* meiotic cells.

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Peer review under responsibility of Ain Shams University.

http://dx.doi.org/10.1016/j.ejmhg.2015.11.002

1. Introduction

Cancer is one of the leading causes of death worldwide and accounted for 8.2 million deaths in 2012 [1]. Depending on the type and stage of cancer, treatments to eradicate the tumor or slow its growth include some combination of surgery, radiation therapy and chemotherapy [2]. Recent alternative targeted therapies are employed namely hyperthermia, hormone therapies, signal transduction inhibitors, gene expression modulator, apoptosis inducer, angiogenesis inhibitor, immunotherapies and toxin delivery molecules [3,4]. Hyperthermia (thermal therapy or thermotherapy) is a type of cancer treatment in which body tissue is exposed to high temperatures (range between 41 °C and 45 °C) to damage and kill cancer cells. It is a good therapeutic tool for non-invasive cancer therapy and is being employed along with traditional radiotherapy, chemotherapy and combination of both (triple modality) [5]. It has also been observed that hyperthermia allows clinicians to reduce doses of anticancer drugs and radiations administered to patients. The reduction of the doses helps, consequently, the reduction of anticancer therapy side effects [6]. Therefore, hyperthermia aims at improving the results of the conventional treatment strategies within a framework of multi-model treatments.

Working with anti-cancerous agents, Scientists have noticed the protection of cells to lethal dose, when these are pre-exposed to low doses. This has come to be known as 'adaptive response' [7] which refers to the ability of cells or organisms to better resist the damaging effects of toxic agent when first pre exposed to a lower dose. When treatment with anti-neoplastic drugs is pursued over a long period, depending on the doses employed, adaptive response, if induced in the cells and tissues involved, can modify the efficacy of the treatment leading to drug or radio-resistance [8,9]. The timing of heat exposure, before or after the ultra violet (UV) or N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) treatments, had no effect on the result in cases of cytotoxicity and mutagenesis [10]. Contrarily, the mild hyperthermia (41 °C for 1 h) can induce adaptation to cytogenetic damages caused by subsequent mutagenic agents [11–14]. Studies with hyperthermia showed that it caused radiosensitization or chemosensitization [15,16]. It is clear from the published data; that there are contradictory reports about the action of hyperthermia and induction of adaptive response by hyperthermia in combination with mutagen. Even though, a few reports are available on the adaptive response in mouse, *Poecilocerus pictus*, *Drosophila*, plant (*Vicia faba*) and human test systems [17–24] using alkylating agents, the influence of hyperthermia has not been analyzed. Further *P. pictus* has been employed as a model insect *in vivo* system to understand the cytogenetical effects [17,24]. The diploid numbers of chromosome complements are 19 in males and 20 in females, which are large in size. Furthermore, cells showing all the meiotic stages are available in large numbers for cytological scrutiny. Hence, in the present investigations, an attempt has been made to understand the influence of hyperthermia on Ethyl methanesulfonate (EMS) induced adaptive response in meiotic cells of the grasshopper *P. pictus*.

2. Materials and methods

2.1. Chemicals

The monofunctional alkylating agent Ethyl methanesulfonate (EMS: CAS No. 62-50.0), an ethylating agent obtained from Sigma Chemical Company, USA was used.

2.2. P. pictus

Male grasshoppers weighing 2.5–3 g were collected from the environs of Mysore city and these were maintained in the laboratory for 2–3 days until use.

2.3. Selection of chemical doses

In order to understand the adaptive response, the conditioning and challenging doses of clastogen have to be selected. The conditioning and challenging doses of EMS were established in previous experiments with *P. pictus* [17,25]. The same doses such as 0.03 M and 0.12 M EMS were used in the present study as conditioning (L) and challenging (H) doses respectively.

2.4. Hyperthermia

Pilot toxicity studies were carried out to select the temperatures (hyperthermia) and the time of exposure in the present study. The grasshoppers were placed in the small cages and hyperthermic exposure was carried out using BOD (biological oxygen demand) incubator. Initial experiments were carried out by subjecting grasshoppers to various temperatures ranging from 38 °C to 45 °C with different times of exposure such as 10, 15, 30 and 45 min. The higher temperatures of 40 °C and 45 °C with exposure time of 15 and 30 min were selected in the present study. The effective hyperthermic temperatures were chosen by understanding the mortality and frequency of chromosomal anomalies produced.

2.5. Treatment schedules

EMS was dissolved in 0.4% NaCl solution. 50 µL of the fixed concentration of the chemical agent was injected into the abdomen of the animal between 3rd and 4th segments. Each time freshly prepared solution of agent was used.

(i) *Control:* The control group of grasshoppers received 50 µL of 0.4 % NaCl solution only.
(ii) *Hyperthermia (HT):* The grasshoppers were exposed to 40° and 45 °C for 15 or 30 min respectively.
(iii) *EMS treatment:* In this treatment schedule, grasshoppers were treated with conditioning (L) or challenging (H) doses of EMS.
(iv) *Combined treatment of EMS:* The previous studies [17,25] have shown that the combined treatment of conditioning and challenging doses of clastogen (EMS) with 2 h time lag (TL) between them offered appreciable protection in meiotic cells of *P. pictus*. Hence, in the present

experiments, the same 2 h TL between conditioning and challenging dose of EMS (L-2 h-H) was employed to understand the occurrence of adaptive response.

(v) *Pre-treatments of hyperthermia:* Grasshoppers were subjected to hyperthermia 2 or 4 h prior to conditioning dose of EMS and then they were challenged with same clastogen after 2 h.
   (1) [HT [40 °C-15 min]-2 h-L-2 h-H].
   (2) [HT [40 °C-30 min]-2 h-L-2 h-H].
   (3) [HT [45 °C-15 min]-2 h-L-2 h-H].
   (4) [HT [45 °C-30 min]-2 h-L-2 h-H].
   (5) [HT [40 °C-15 min]-4 h-L-2 h-H].
   (6) [HT [40 °C-30 min]-4 h-L-2 h-H].
   (7) [HT [45 °C-15 min]-4 h-L-2 h-H].
   (8) [HT [45 °C-30 min]-4 h-L-2 h-H].

(vi) *Inter-treatments of hyperthermia:* The grasshoppers were subjected to hyperthermia in between the conditioning and challenging treatment of EMS. Grasshoppers were exposed to hyperthermia for one hour after conditioning dose of EMS and one hour later they were challenged with challenging dose of the same clastogen with 15 or 30 min time of hyperthermia.
   (1) [L-1 h-HT [40 °C-15 min]-1 h-H].
   (2) [L-1 h-HT [40 °C-30 min]-1 h-H].
   (3) [L-1 h-HT [45 °C-15 min]-1 h-H].
   (4) [L-1 h-HT [45 °C-30 min]-1 h-H].

(vii) *Post-treatments of hyperthermia:* In this schedule grasshoppers were exposed to hyperthermia, 2 or 4 h after combined treatment (L-2 h-H) of EMS.
   (1) L-2 h-H-2 h-HT [40 °C-15 min].
   (2) L-2 h-H-2 h-HT [40 °C-30 min].
   (3) L-2 h-H-2 h-HT [45 °C-15 min].
   (4) L-2 h-H-2 h-HT [45 °C-30 min].
   (5) L-2 h-H-4 h-HT [40 °C-15 min].
   (6) L-2 h-H-4 h-HT [40 °C-30 min].
   (7) L-2 h-H-4 h-HT [45 °C-15 min].
   (8) L-2 h-H-4 h-HT [45 °C-30 min].

(viii) *Cross adaptation:* In this set of experimental schedule, grasshoppers were exposed to hyperthermia first and then the same animals were challenged after 2 h with challenging dose of EMS.
   (1) [HT [40 °C-15 min]-2 h-H].
   (2) [HT [40 °C-30 min]-2 h-H].
   (3) [HT [45 °C-15 min]-2 h-H].
   (4) [HT [45 °C-30 min]-2 h-H].

All the treated and control animals were maintained on fresh *Calotropis* leaves in the respective cages. The grasshoppers were sacrificed at 12, 24, 36 or 48 h of recovery times. A minimum of three experiments were carried out. A total of 12 animals were used for each treatment schedule.

2.6. Meiotic chromosome preparation

Chromosome preparations were made by following the procedure of Riaz Mahmood and Vasudev [17]. In brief, Grasshoppers were sacrificed by decapitation. The testes were removed from the abdomen and fixed in methanol/acetic acid (3:1 v/v). Three changes of the fixative for 15 min in each were given to the material. Meanwhile, the testes were cleaned by removing the fat and tracheae (respiratory organ of insect). These testes were then kept in absolute methanol for 10 min. They were then transferred and preserved in 70% ethyl alcohol until further use. Each tubule of the testes was washed using distilled water at the time of temporary chromosome preparation. They were then transferred to mordant, 4 % iron alum (Ferric ammonium sulfate). After 20 min, these were stained using Heidenhain's hematoxylin stain for 30 min. The stained tubules were washed using distilled water and 3–4 tubules were placed on a clean, non-greasy, micro slide with few drops of freshly prepared 45% acetic acid. Cover glass was placed after 5 min on the tubules and gently pressed using blotting paper. The cover glass was sealed with wax.

2.7. Chromosome analysis

Coded slides from grasshoppers belonging to various treatment regimen were screened to score the chromosomal anomalies in the different stages of meiosis such as metaphase I, anaphase I, metaphase II and anaphase II. The chromosomal anomalies *viz*., stickiness, stickiness and clumping, fragments, bridges, pseudobridges and laggards, were recorded. In each grasshopper a minimum of 500 cells in each meiotic stage and a total of 2000 cells were scored. Thus, a total of 24,000 meiotic cells in 12 grasshoppers were scored per each treatment schedule.

2.8. Statistical analysis

The difference that exists among the mean differences in the treatment groups was analyzed using the Duncan multiple comparison post hoc test using the SPSS software (version 16.0). The Duncan post hoc test makes pairwise comparisons using a stepwise order of comparisons among the treatment groups.

3. Results

The frequencies of different chromosomal anomalies such as stickiness, stickiness and clumping, fragments, bridges, pseudobridges and laggards that were observed after different treatments are given in Table 1a. Stickiness and stickiness and clumping were found to be prominent in EMS treatment compared to that of controls and hyperthermia. Both conditioning and challenging doses induced significant anomalies at different temperatures (40 °C and 45 °C) exposed to different durations (15 and 30 min). Combined treatment with 2 h TL between them resulted in 44–50% reduction of chromosomal anomalies which is significant compared to that of additive effect at 12, 24, 36 and 48 h recovery times (Table 1b).

Pre treatment of hyperthermia to EMS exposed cells resulted in significant reduction of the range of 59 to 67% chromosomal anomalies compared to that of additive effects (Table 2, $p < 0.05$). It is also evident when temperatures of 40 °C and 45 °C for 15 and 30 min with 2 h and 4 h time intervals were used (Fig. 1). The frequencies of anomalies were significantly reduced when hyperthermia was given between

Table 1a Frequency (%) of individual chromosomal anomalies (mean ± SE) observed after hyperthermia (HT) or Ethyl methanesulfonate (EMS) treatment in meiotic cells of *P. pictus* at 12 h recovery times (RTs).

Treatment Groups	Metaphase I			Anaphase I			Metaphase II			Anaphase II			Total damage
	St	St & Cl	Fr	Br	Lag	Fr	St	St &Cl	Fr	PB	Lag	Fr	
Control	1.35 ± 0.056	–	–	0.05 ± 0.029	1.66 ± 0.034	–	3.58 ± 0.172	0.45 ± 0.055	–	–	–	–	7.09 ± 0.249[a]
HT-40 °C-15 min	1.63 ± 0.059	–	–	0.05 ± 0.039	1.59 ± 0.031	–	4.05 ± 0.174	0.57 ± 0.031	–	–	–	–	7.89 ± 0.174[a]
HT-40 °C-30 min	1.60 ± 0.037	–	–	0.10 ± 0.048	1.85 ± 0.070	–	3.58 ± 0.218	0.54 ± 0.056	–	–	–	–	7.67 ± 0.270[a]
HT-45 °C-15 min	1.65 ± 0.022	–	–	0.04 ± 0.021	2.15 ± 0.044	–	3.61 ± 0.086	0.53 ± 0.043	–	–	–	–	7.98 ± 0.111[a]
HT-45 °C-30 min	1.45 ± 0.031	–	–	0.03 ± 0.018	1.90 ± 0.038	–	4.07 ± 0.030	0.44 ± 0.020	–	–	–	–	7.89 ± 0.066[a]
EMS-L	4.98 ± 0.117	1.21 ± 0.036	1.59 ± 0.041	1.29 ± 0.028	0.74 ± 0.104	–	18.60 ± 0.150	3.52 ± 0.069	2.84 ± 0.090	2.28 ± 0.047	0.40 ± 0.037	–	37.45 ± 0.318[b]
EMS-H	8.10 ± 0.039	7.88 ± 0.070	0.16 ± 0.024	4.39 ± 0.055	1.48 ± 0.065	0.51 ± 0.015	29.47 ± 0.143	28.94 ± 0.211	1.32 ± 0.039	2.17 ± 0.019	1.30 ± 0.029	–	84.41 ± 1.271[d]
L-2 h-H	5.83 ± 0.113	5.83 ± 0.145	0.95 ± 0.088	1.13 ± 0.027	0.69 ± 0.032	–	28.42 ± 0.175	15.57 ± 0.115	0.43 ± 0.061	1.10 ± 0.086	1.08 ± 0.032	0.02 ± 0.012	67.54 ± 0.963[c]

Pooled data from three independent experiments; minimum of 500 cells in each meiotic stage per dose were scored; minimum of 4 grasshoppers per experiment were used.
Values with same superscripts are not significant ($p > 0.05$); Values with different superscripts are significantly different from one another ($p < 0.05$) according to Duncan Post hoc test.
St: stickiness; St&Cl: stickiness and clumping; Fr: fragments; Br: bridges; Lag: laggards; PB: pseudo bridges.
L: conditioning dose; H: challenging dose; HT: hyperthermic treatment.

Table 1b Percentage of chromosomal anomalies (mean ± SE) observed after HT or EMS treatment in meiotic cells of *P. pictus* at different RTs.

Treatment groups	% Chromosomal anomalies at different RT (in h)			
	12	24	36	48
Control	7.09 ± 0.249[a]	7.10 ± 0.125[a]	7.12 ± 0.071[a]	7.17 ± 0.096[a]
HT-40 °C-15 min	7.89 ± 0.174[a]	7.66 ± 0.199[a]	7.55 ± 0.190[a]	7.52 ± 0.150[a]
HT-40 °C-30 min	7.67 ± 0.270[a]	7.55 ± 0.097[a]	7.41 ± 0.166[a]	7.35 ± 0.167[a]
HT-45 °C-15 min	7.98 ± 0.111[a]	7.81 ± 0.149[a]	7.75 ± 0.093[a]	7.67 ± 0.094[a]
HT-45 °C-30 min	7.89 ± 0.066[a]	7.70 ± 0.056[a]	7.67 ± 0.212[a]	7.58 ± 0.088[a]
EMS-L	37.45 ± 0.318[b]	32.83 ± 0.306[b]	31.86 ± 0.252[b]	28.16 ± 0.208[b]
EMS-H	84.41 ± 1.271[d]	78.92 ± 0.264[d]	76.14 ± 0.334[d]	68.22 ± 0.497[d]
L-2 h-H	67.54 ± 0.963[c]	60.39 ± 0.207[c]	53.62 ± 0.432[c]	51.17 ± 0.463[c]
% Reduction	44.51 ± 1.001*	45.94 ± 0.352*	50.34 ± 0.441*	46.89 ± 0.544*

Note: Pooled data from three independent experiments; minimum of 500 cells in each meiotic stage per dose were scored; minimum of 4 grasshoppers per experiment were used. Values with same superscripts are not significant ($p > 0.05$); values with different superscripts are significantly different from one another ($p < 0.05$) according to Duncan Post hoc test.
Individual chromosomal anomalies were scored as per Table 1a and pooled to make data concise, thus the percentage anomalies for different recovery times are given in this table.
Calculation of percent reduction: (A) Additive effect: sum of chromosomal anomalies observed in both conditioning (L) and challenging (H) dose (L + H); (B) combined Effect: chromosomal anomalies observed in combined treatment of conditioning and challenging doses with 2 h time lag (L-2 h-H); percentage of reduction (C) was calculated by using formula: $C = (B/A * 100) - 100$.
* Values are significant compared to additive effect ($p < 0.05$).

Table 2 Percentage of chromosomal anomalies (mean ± SE) observed after pretreatment of HT to combine (conditioning and challenging) doses of EMS treated meiotic cells of *P. pictus* at different RTs.

Treatment Groups	% Chromosomal anomalies at different RT (in h)			
	12	24	36	48
HT-40 °C-15 min-2 h-L-2 h-H	47.49 ± 0.160[f]	45.42 ± 0.196[f]	39.35 ± 0.226[f]	38.32 ± 0.117[f]
HT-40 °C-30 min-2 h-L-2 h-H	43.30 ± 0.084[d]	42.19 ± 0.098[d]	37.79 ± 0.124[e]	35.35 ± 0.156[d]
HT-45 °C-15 min-2 h-L-2 h-H	46.33 ± 0.219[ef]	45.36 ± 0.157[f]	38.01 ± 0.141[e]	37.82 ± 0.175[f]
HT-45 °C-30 min-2 h-L-2 h-H	43.02 ± 0.182[d]	42.14 ± 0.136[d]	37.00 ± 0.139[e]	35.13 ± 0.134[d]
HT-40 °C-15 min-4 h-L-2 h-H	45.33 ± 0.118[e]	43.03 ± 0.203[e]	37.02 ± 0.124[e]	36.01 ± 0.101[e]
HT-40 °C-30 min-4 h-L-2 h-H	42.83 ± 0.120[d]	42.01 ± 0.130[d]	36.13 ± 0.154[d]	35.03 ± 0.099[d]
HT-45 °C-15 min-4 h-L-2 h-H	44.01 ± 0.164[d]	42.02 ± 0.186[d]	36.03 ± 0.087[d]	35.02 ± 0.094[d]
HT-45 °C-30 min-4 h-L-2 h-H	41.05 ± 0.093[c]	40.04 ± 0.123[c]	34.68 ± 0.055[c]	34.00 ± 0.082[c]

Pooled data from three independent experiments; minimum of 500 cells in each meiotic stage per dose were scored; minimum of 4 grasshoppers per experiment were used.
Values with same superscripts are not significant ($p > 0.05$); values with different superscripts are significantly different from one another ($p < 0.05$) according to Duncan Post hoc test.
Individual chromosomal anomalies were scored as per Table 1a and pooled to make data concise, thus the percentage anomalies for different recovery times are given in this table.

conditioning and challenging doses of EMS at all tested recovery times ($p < 0.05$; Table 3). The percentage reduction of chromosomal anomalies is between 56 and 63%, which is significant (Fig. 2). There is a significant decrease in anomalies in post treatment of hyperthermia compared to combined treatment of EMS ($p < 0.05$; Table 4). The percentage reduction of chromosomal anomalies is between 47 and 55% (Fig. 3). The treatment of hyperthermia prior to challenging dose (i.e. hyperthermia + challenging dose) reduced chromosomal anomalies significantly compared to challenging dose at all recovery times tested (Table 5; $p < 0.05$). The reduced yield of chromosomal anomalies is around 32% at different temperatures and RTs (Fig. 4).

Although reductions of chromosomal anomalies were quite different at different temperatures and time intervals, more reductions of chromosomal anomalies were detected at 45 °C than at 40 °C in all the pre, inter, post and cross adaptation treatment schedule groups. This is also true for time intervals in that 2 h time interval noticed high anomaly frequency than at 4 h time interval at all recovery times (Table 1a–5 and Figs. 1–4).

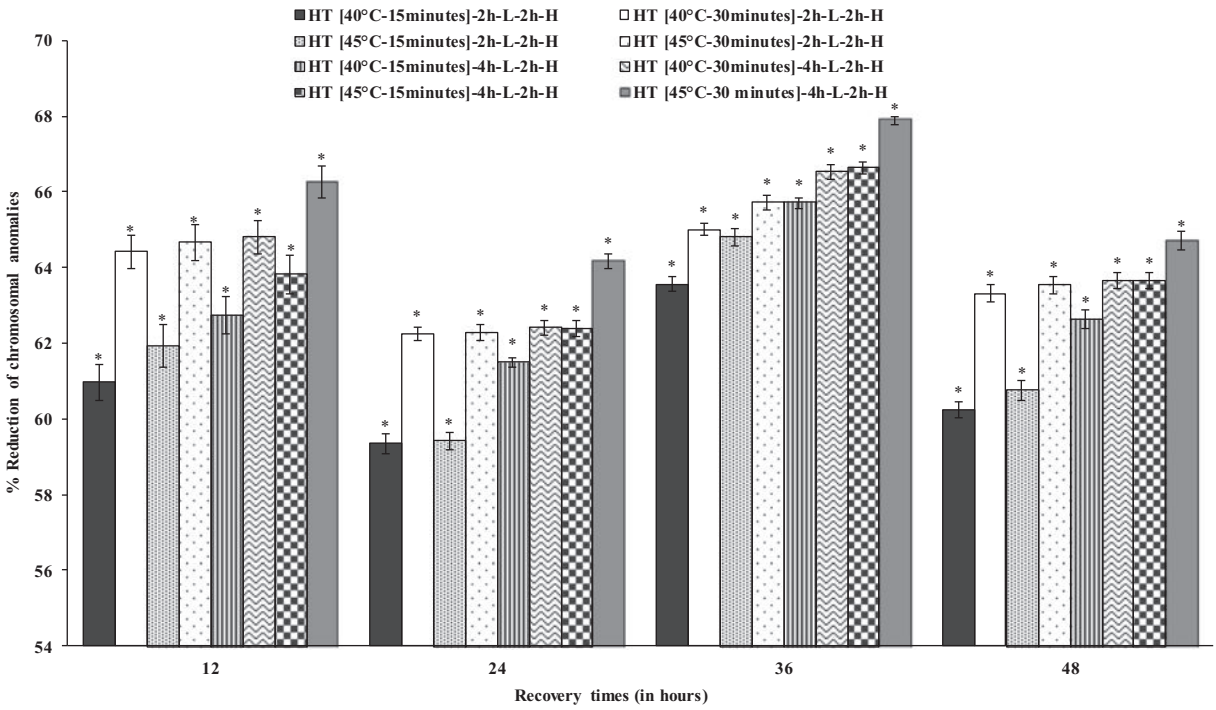

Figure 1 Reduction (%) of chromosomal anomalies (mean ± SE) observed after pre treatment of hyperthermia (HT) compared to additive effect of Ethyl methanesulfonate (EMS) at different RTs in *P. pictus*. *Note:* Additive effect: sum of chromosomal anomalies observed in both conditioning and challenging doses (L + H); * values are significant compared to additive effect ($p < 0.05$).

Table 3 Percentage of chromosomal anomalies (mean ± SE) observed after inter treatment of HT between conditioning and challenging doses of EMS in meiotic cells of *P. pictus* at different RTs.

Treatment Groups	% Chromosomal anomalies at different RT (in h)			
	12	24	36	48
L-1 h-HT-40 °C-15 min-1 h-H	48.04 ± 0.154^{e}	46.04 ± 0.117^{f}	44.78 ± 0.105^{f}	42.22 ± 0.101^{d}
L-1 h-HT-40 °C-30 min-1 h-H	47.26 ± 0.068^{de}	45.02 ± 0.083^{e}	43.18 ± 0.082^{de}	42.03 ± 0.097^{d}
L-1 h-HT-45 °C-15 min-1 h-H	46.12 ± 0.093^{cd}	44.00 ± 0.052^{d}	42.29 ± 0.075^{d}	41.01 ± 0.112^{c}
L-1 h-HT-45 °C-30 min-1 h-H	45.29 ± 0.125^{c}	43.00 ± 0.067^{c}	41.02 ± 0.064^{c}	40.57 ± 0.066^{c}

Pooled data from three independent experiments; minimum of 500 cells in each meiotic stage per dose were scored; minimum of 4 grasshoppers per experiment were used.
Values with same superscripts are not significant ($p > 0.05$); values with different superscripts are significantly different from one another ($p < 0.05$) according to Duncan Post hoc test.
Individual chromosomal anomalies were scored as per Table 1a and pooled to make data concise, thus the percentage anomalies for different recovery times are given in this table.

4. Discussion

4.1. Individual chromosome anomalies

Among different types of meiotic chromosomal anomalies observed, the chromosome stickiness and stickiness and clumping are the prominent ones with high frequencies (Table 1a). In stickiness and clumping chromosome complement stuck together and formed irregular masses and in the extreme clump the individuality of chromosome was lost. Stickiness has been reported to be induced by a variety of chemicals in grasshopper spermatocytes [26–28]. Various biochemical views on the stickiness and clumping have been put forth by many workers. Stickiness results from the breakdown of chromosomal nucleic acid into the depolymerized and fluid state [29], the dissociation of nucleic acid into the nuclear sap [30], high proteolytic activity [31] and excess of histone might cross link DNA in the neighboring strands [32]. On the basis of electron microscopic examination it was reported that mammalian sticky chromosome and *Allium cepa* root tip induced by chemicals possess fine fibrous connections between chromosomes and supposed that these are chromatid fibers [27]. From this, it can be concluded that chromosome stickiness is a chromatid type of aberration.

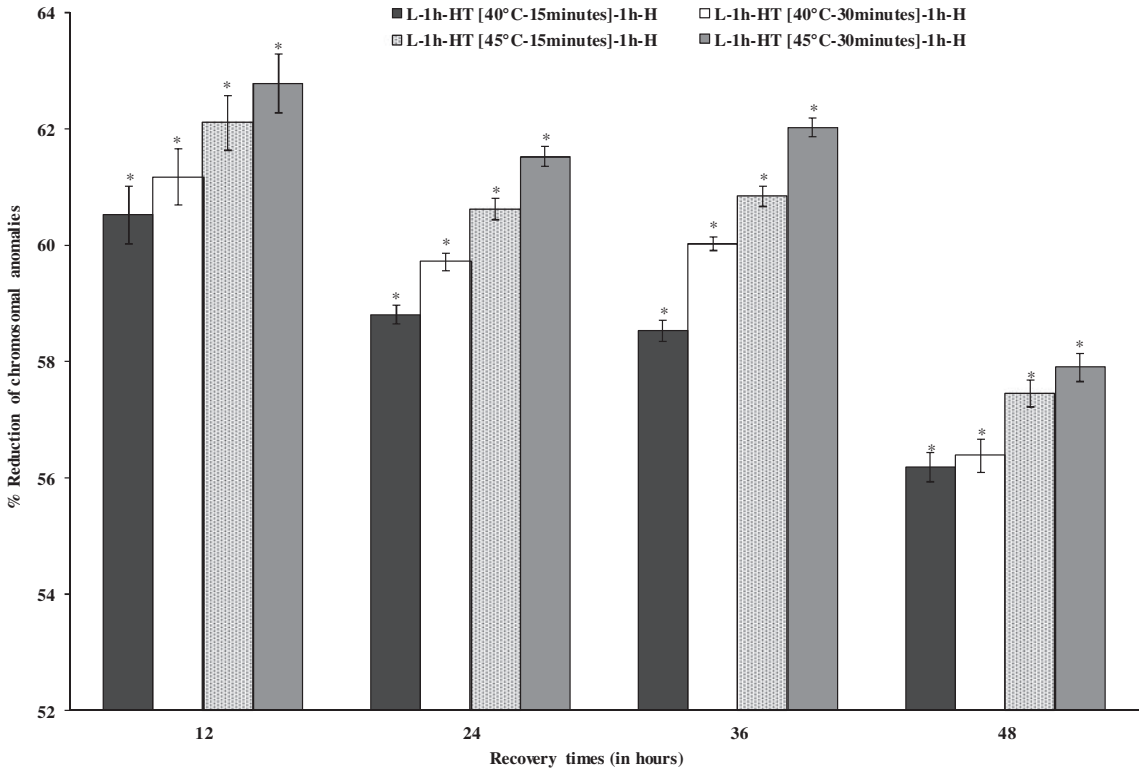

Figure 2 Reduction (%) of chromosomal anomalies (mean ± SE) observed after inter treatment of HT compared to additive effect of EMS at different RTs in *P. pictus*. *Note:* Additive effect: sum of chromosomal anomalies observed in both conditioning and challenging doses (L + H); * values are significant compared to additive effect ($p < 0.05$).

Table 4 Percentage of chromosomal anomalies (mean ± SE) observed after post treatment of HT to combined (conditioning and challenging) EMS dosed in meiotic cells of *P. pictus* at different RTs.

Treatment Groups	% Chromosomal anomalies at different RT (in h)			
	12	24	36	48
L-2 h-H-2 h-HT-40 °C-15 min	60.52 ± 0.052^{f}	59.16 ± 0.085^{i}	52.13 ± 0.094^{h}	50.57 ± 0.112^{gh}
L-2 h-H-2 h-HT-40 °C-30 min	59.22 ± 0.044^{de}	58.31 ± 0.075^{h}	51.46 ± 0.052^{gh}	49.54 ± 0.184^{ef}
L-2 h-H-2 h-HT-45 °C-15 min	59.49 ± 0.051^{ef}	58.95 ± 0.050^{i}	52.26 ± 0.958^{h}	50.11 ± 0.076^{fg}
L-2 h-H-2 h-HT-45 °C-30 min	58.04 ± 0.076^{d}	57.30 ± 0.028^{g}	50.70 ± 0.133^{fg}	48.52 ± 0.295^{d}
L-2 h-H-4 h-HT-40 °C-15 min	59.03 ± 0.028^{de}	56.29 ± 0.067^{f}	50.11 ± 0.088^{ef}	49.33 ± 0.060^{e}
L-2 h-H-4 h-HT-40 °C-30 min	58.50 ± 0.042^{de}	55.50 ± 0.058^{e}	49.51 ± 0.078^{de}	47.95 ± 0.068^{d}
L-2 h-H-4 h-HT-45 °C-15 min	57.94 ± 0.031^{d}	54.42 ± 0.053^{d}	49.06 ± 0.065^{d}	48.07 ± 0.226^{d}
L-2 h-H-4 h-HT-45 °C-30 min	56.09 ± 0.057^{c}	53.25 ± 0.041^{c}	48.06 ± 0.058^{c}	46.46 ± 0.053^{c}

Pooled data from three independent experiments; minimum of 500 cells in each meiotic stage per dose were scored; minimum of 4 grasshoppers per experiment were used.
Values with same superscripts are not significant ($p > 0.05$); values with different superscripts are significantly different from one another ($p < 0.05$) according to Duncan Post hoc test.
Individual chromosomal anomalies were scored as per Table 1a and pooled to make data concise, thus the percentage anomalies for different recovery times are given in this table.

4.2. EMS induced adaptive response in P. pictus

The decrease in chromosomal anomalies after combined treatments in comparison with challenge or additive doses must be due to the induction of protective function (adaptive response), by low dose of EMS in meiotic cells of *P. pictus* (Table 1b). Similar results have been recorded in the induction of adaptive response in *V. faba*, *P. pictus* and human lymphocytes by alkylating agents [17,19,33–35]. The results of the present investigations, together with previous investigations indicate that the factors involved in the adaptive response may be very complex in eukaryotic systems. Most of the studies revealed in plants and human lymphocytes *in vitro* that clastogenic adaptation depends on unimpaired protein synthesis [37] and on metabolic state of the cells. These findings indicate the presence of inducible protective functions (possible repair

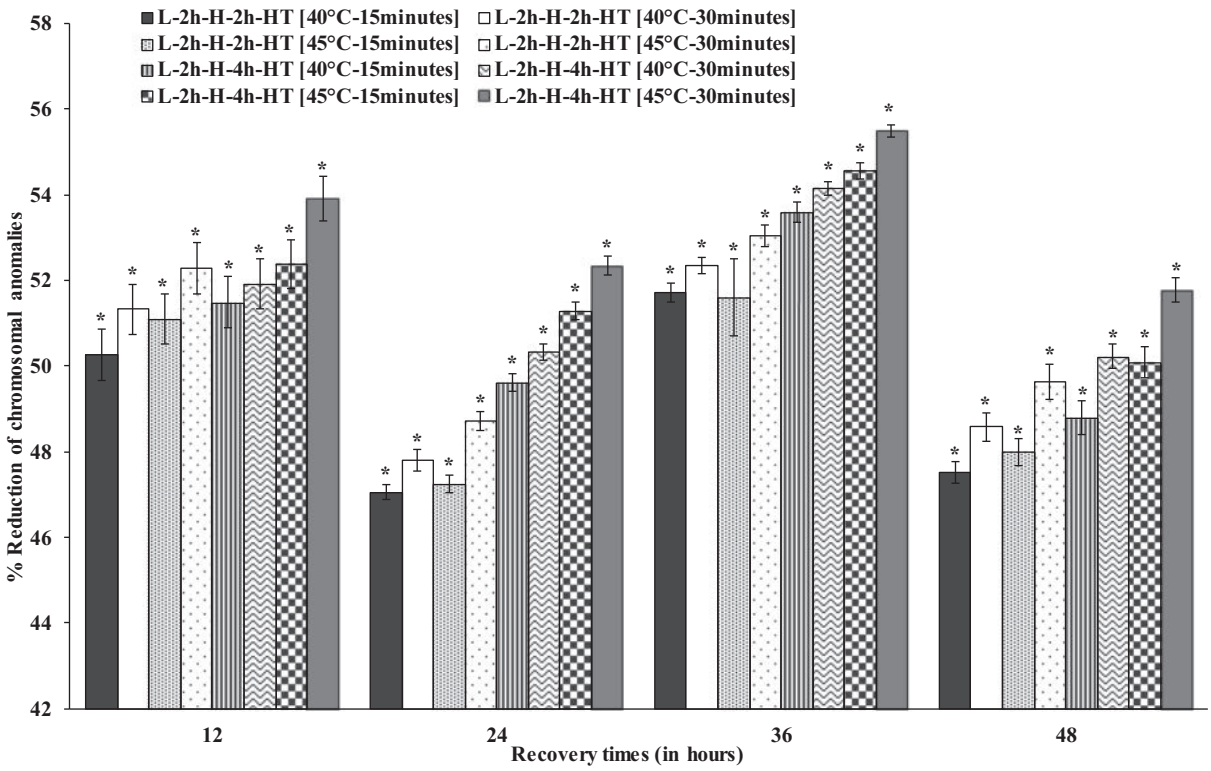

Figure 3 Reduction (%) of chromosomal anomalies (mean ± SE) observed after post treatment of HT compared to additive effect of EMS at different RTs in *P. pictus*. *Note:* Additive effect: sum of chromosomal anomalies observed in both conditioning and challenging doses (L + H); * values are significant compared to additive effect ($p < 0.05$ level).

Table 5 Percentage of chromosomal anomalies (mean ± SE) observed in meiotic cells of *P. pictus* treated with HT and challenging with high dose of EMS at different RTs.

Treatment Groups	% Chromosomal anomalies at different RT (in h)			
	12	24	36	48
HT-40 °C-15 min-2 h-H	58.14 ± 0.117^{e}	56.20 ± 0.069^{e}	52.56 ± 0.086^{f}	49.06 ± 0.533^{ef}
HT-40 °C-30 min-2 h-H	57.26 ± 0.024^{de}	55.39 ± 0.067^{d}	51.29 ± 0.033^{e}	48.13 ± 0.073^{de}
HT-45 °C-15 min-2 h-H	56.05 ± 0.075^{cd}	54.74 ± 0.103^{c}	49.74 ± 0.043^{d}	47.06 ± 0.039^{cd}
HT-45 °C-30 min-2 h-H	55.05 ± 0.090^{c}	54.26 ± 0.300^{c}	48.49 ± 0.079^{c}	46.17 ± 0.050^{c}

Pooled data from three independent experiments; minimum of 500 cells in each meiotic stage per dose were scored; minimum of 4 grasshoppers per experiment were used.
Values with same superscripts are not significant ($p > 0.05$); values with different superscripts are significantly different from one another ($p < 0.05$) according to Duncan Post hoc test.
Individual chromosomal anomalies were scored as per Table 1a and pooled to make data concise, thus the percentage anomalies for different recovery times are given in this table.

activities). Even though the adaptive repair system in bacteria is well demonstrated [36], the situation as to the existence of such a mechanism in mammalian cells is not yet clear. Furthermore underlying mechanisms of clastogenic adaptation in mammalian *in vivo* systems are presently unknown that too in meiotic cells.

In all the treatments, different recovery times (fixed times) have been employed after the challenge treatment. If one recovery time was selected, then one would have argued that the reduced anomalies' yields observed after different treatments are due to the effects of pre treatment in the cell cycle. To exclude this argument in the present investigations, different recovery times were selected to study the induction of protection in different cell population in *P. pictus*. It was suggested that the response ceases after the third mitosis of adapted cells, due to a dilution of the repair system as the cells divide over subsequent cell cycles [37]. This agrees with earlier reports where it has been fully proved that the decrease in anomaly frequency with increasing culture time reflects a mechanism of mitotic selection of anomalies bearing cells.

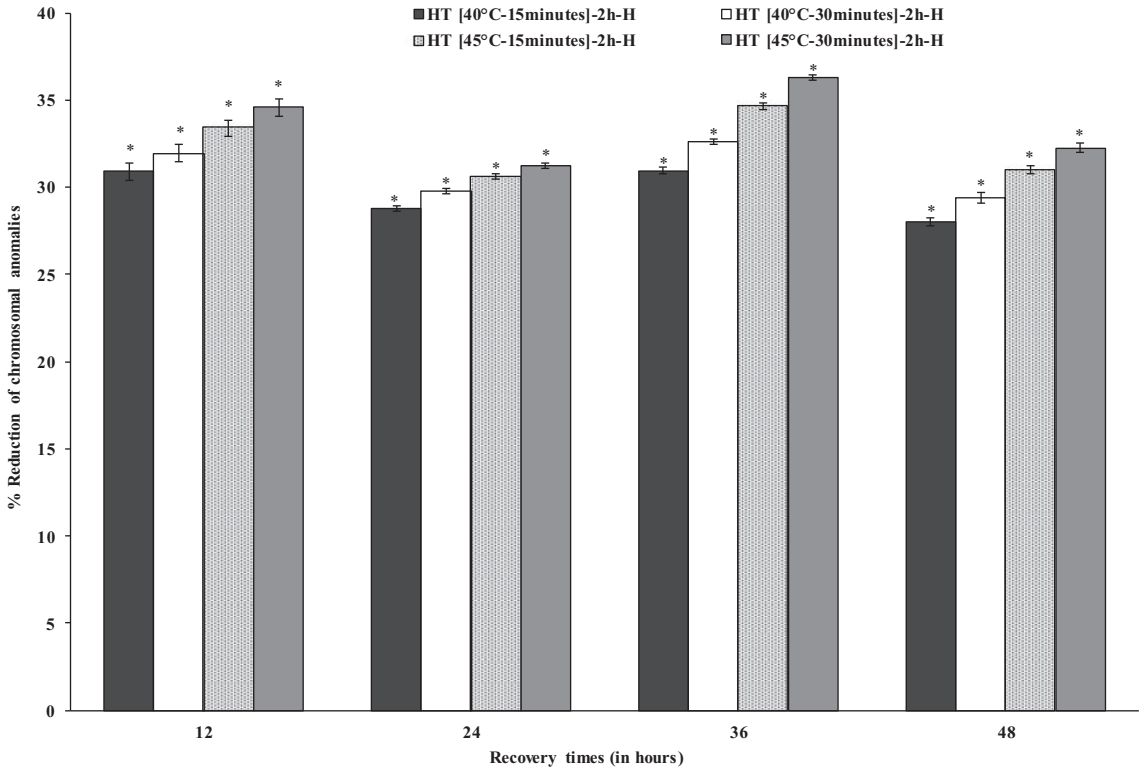

Figure 4 Reduction (%) of chromosomal anomalies (mean ± SE) observed in *P. pictus* treated with HT compared to high dose of EMS at different RTs in *P. pictus*. *Note:* Additive effect: sum of chromosomal anomalies observed in both conditioning and challenging doses (L + H); * values are significant compared to additive effect ($p < 0.05$).

4.3. Influence of hyperthermia pretreatment on EMS induced cytogenetic adaptive response

Hyperthermia of 40 °C or 45 °C could not induce significant anomalies in meiotic cells of *P. pictus* at different time intervals at all recovery times analyzed compared to that of controls and thus it can be said that hyperthermia could not induce any lesions ($p > 0.05$; Table 1b). On par with this, similar observations were made by earlier workers [38,39]. Contrary to these, it has been demonstrated that hyperthermia could induce chromosomal aberrations in *in vitro* Chinese hamster ovary (CHO) cells [40] in He La cells [41] and human A549 cells [42]. Review of literature thus, reveals that there are conflicting reports on the effects of hyperthermia on chromosome on one hand and on the other there are no sufficient reports on the effects of hyperthermia using *in vivo* systems.

The significant decrease of chromosomal anomalies in meiotic cells of *P. pictus* ($p < 0.05$; Table 2), after pretreatment of hyperthermia demonstrates the enhancement of adaptive response by hyperthermia in *in vivo* system. Cai and Jiang [13] working with human lymphocytes have demonstrated that, hyperthermia and adaptive dose + challenging dose with an interval of 6 h reduced the number of chromatid and isochromatid breaks to 30 to 70%. The absence of additive effect after second adaptive dose was hypothesized to be due to the saturation effect of a single adaptive dose [13]. Interestingly, the present results demonstrated that, hyperthermia as the first adaptive dose and EMS as the second adaptive dose induced highly significant adaptation to subsequent challenge dose of the said agent in *P. pictus*. For example combined doses of EMS (L + H) resulted in 60.39% reduction on one hand and 66.33% in combined treatments of hyperthermia + adaptive dose of EMS + high dose of EMS in meiotic cells of *P. pictus* at 24 h recovery time (Fig. 1). These results suggest that, there is more additive or nearly synergistic effects proving that the adaptation induced by hyperthermia involves the different mechanisms compared to chemical adaptation.

The primary heat treatment and heating time for the degree and kinetics of thermotolerance in the treatment of carcinoma is very important. Preheating of the tumors at 43.5 °C for 3.5, 7.5, 15, 30, or 45 min, showed that, both the thermotolerance ratio and the time interval which are necessary to develop thermotolerance ratio increased, both being linear functions of the duration of the preheating time. Maximal thermotolerance was obtained at intervals of 2, 4, 8, 16 and 28 h with thermotolerance ratio of 1.6, 2.2, 3.7, 5.2 and 7.7 respectively [43]. Rieger and Michaelis [44] have shown reduction in maleic hydrazide or triethylenemelamine induced chromatid aberrations in the cells which are pre exposed to heat shock (10 min; 40 °C). They also demonstrated that the protective function of heat shock is a quick response which lasts up to 240 min and suggests that heat shock before clastogen treatment triggers clastogen-specific, protective functions which eventually result in protection against clastogens. Similarly, there was reduction in the chromatid aberrations in *V. faba* seedlings which are pre treated with sub-lethal heat shock (10 min, 40 °C) and then challenged with N-Methyl-N-Nitrosourea (MNU) when compared to challenging treatment of MNU alone [45]. These

evidences indicate the beneficial role of conditioning treatment of heat shock in reducing DNA damages.

4.4. Influence of hyperthermia inter-treatment on EMS induced cytogenetic adaptive response

The inter treatment of hyperthermia (L-1 h-HT-1 h-H) with EMS yielded significantly less frequency of chromosomal anomalies compared to combined treatment (L-2 h-H) indicating the enhancement of adaptive response in *P. pictus* ($p < 0.05$; Table 3). On par with these results, Cai and Jiang [13] working with human lymphocytes in the combination of hyperthermia inter treatment such as (i) adaptive dose (50 mGy X rays) and hyperthermia (0 h, 41 °C for 1 h) + challenging dose (1.5 Gy X rays) (ii) adaptive dose (50 mGy X rays) and hyperthermia (14 h, 41 °C for 1 h) + challenging dose (1.5 Gy X rays) (iii) adaptive dose (50 mGy X rays) and hyperthermia (38 h, 41 °C for 1 h) + challenging dose (1.5 Gy X rays) (iv) adaptive dose (50 mGy X rays) + hyperthermia (42 h, 41 °C for 1 h) + challenging dose (1.5 Gy X rays) together reduced the chromatid and isochromatid breaks of the effects induced by challenge dose alone. Bleomycin (10 mg/kg) given intra peritoneal before heat and then radiation was administered as 5 fractions of 3 Gy resulting in increased growth delay up to 14.5 days in FSaIIC fibrosarcoma tumor cells [46]. As has been discussed in the pre treatments even inter treatment of hyperthermia showed clasto-resistance irrespective of time and temperature (Fig. 2).

4.5. Influence of hyperthermia post treatment on EMS induced cytogenetic adaptive response

In the post treatments of hyperthermia after 2 h or 4 h time interval in *P. pictus* yielded significantly lower frequencies of chromosomal anomalies compared to combined treatments at all recovery times ($p < 0.05$; Table 4). Administering the Bleomycin followed by radiation then hyperthermia as a post treatment, produced 1.5 to 2.5-fold greater tumor cell killing than did radiation-Bleomycin-hyperthermia in FSaIIC fibrosarcoma tumor cell line [46]. Contrary to the present finding post treatments of heat treated cells with Trenimone (trifunctional alkylating agent) have synergetic effects on the frequency of chromatid intra and inter changes and this effect can be seen when the cells are recovered after 16, 18 or 22 h in the presence of BrdU [47]. The present results show that adaptive dose + challenge along with hyperthermia of different temperature and time intervals can induce the adaptation to cytogenetic damage in *P. pictus*. Unlike pre and inter treatments, in these schedules 45 °C induced more or less same adaptation at 40 °C in *P. pictus* at all recovery times (Fig. 3).

4.6. Influence of hyperthermia on EMS induced cytogenetic cross adaptive response

It is well established that chemotherapy in most cases has the greatest effect when administered during the heating interval [48]. When heat is given prior to the administration of the drugs/radiation, it can actually increase the resistance/adaptation of the cell/tissue/organisms to that particular therapeutic agent. Thus, in the present study, when *P. pictus* was exposed to hyperthermia first and then the same animals were challenged with high dose of EMS it yielded significantly reduced chromosomal anomalies compared to that of combined treatment ($p < 0.05$; Table 5). This suggests that there is cross adaptation in meiotic cells. Similarly, an adaptive response to mild hyperthermia was first observed in *Escherichia coli* by Cairns and his collaborators [49] and then human lymphocytes [50]. A mild heat shock induced a cross-protection against lethal salt stress in bacteria *Bacillus subtilis* [51]. When CHO cells preheated for varying times at 43 °C, cells became progressively more resistant to subsequent Adramycine treatment [52]. Exposure to 43 °C with actinomycin D for more than 30 min or preheating at 43 °C before drug exposure, both reduced the cytotoxicity of actinomycin D [53]. The EMT6 mouse tumor cells were preheated for 3 h at 40 °C along with cytotoxic agents that produced measurable protection (thermal tolerance) to subsequent treatment for 1 h at 43 °C. This preheat treatment was further found to reduce cell killing by bleomycin (BLM) and 1,3-bis(2-chlorethyl)nitrosourea (BCNU) (drug tolerance) present during 1 h at 43 °C [54]. Heat prior to the administration of the drugs such as adriamycin or actinomycin D can actually increase the resistance of the cell to the chemotherapeutic agents [48]. Vasudev and Obe [47] have demonstrated the pretreatment of CHO cells with heat (46 °C for 6 min) led to a reduction of Alu-I restriction endonuclease induced chromosome aberrations.

The results at the end of each exposure period also showed that there is a significant more production of anomalies at 40 °C compared to 45 °C in *P. pictus* at all recovery times (Figs. 1–4) tested. When hyperthermia was pre treated with challenge dose of radiation (X-rays) it resulted in significantly reduced number of chromatid and isochromatid breaks compared to challenge dose at different time intervals of 0, 14, 38 and 42 h [13]. Heat shock treatment for 10 or 30 min 1 or 2 h prior to maleic hydrazide (MH) [55] or MNU [45] resulted in a significant decrease in the percentage of metaphases with chromatid aberrations at different recovery times tested. Even though similar results were obtained when triethylene melamine (TEM) instead of MH was used; prolongation of time interval i.e. 2 h instead of 1 h between heat shock and TEM resulted in aberrations yield approaching the control value. A shorter heat shock (10 min) proved to be insufficient to lower the TEM effects over the different recovery times tested. In the present investigations, heat treatments for 15 to 30 min applied 2 h prior to EMS in *P. pictus*; resulted in a significant decrease in chromosomal anomalies in *P. pictus* for the whole range of recovery times tested (Tables 2–5). Thus, heat treatment prior to EMS applications reduced the clastogenic activity of both the agents efficiently with same time span.

Early experiments with human lymphocytes revealed that full adaptation to ionizing radiation did not occur until 4 to 6 h after the adapting dose [56]. This observation is generally explained by the necessity of protein synthesis for the adaptation to occur. Recent observations further support this, although the time necessary for adaptation appears to be variable [21]. Thus, an adapting dose was only capable of reducing the frequency of neoplastic transformation when the cells were left in contact inhibition for 24 h before plating [57,58]. Moreover, in mammalian cell culture systems, a low dose of 0.02 Gy delivered 5 h before a challenged dose significantly enhanced the survival rate and resulted in a reduction of induced chromosomal aberrations [59].

5. Conclusion

Hyperthermic treatment could not induce significant chromosomal anomalies compared to that of control at different recovery times in *P. pictus*. The pre, inter and post treatments of hyperthermia to combined treatments have significantly reduced the yield of chromosomal anomalies compared to challenge dose of EMS in *in vivo* test system analyzed. When *P. pictus* was exposed to hyperthermia first and then the same animals were challenged with high dose of EMS, the results have revealed that there are significantly reduced chromosomal anomalies compared to combined treatment at different recovery times. Thus, the overall data of the present study demonstrate that there is enhanced influence of hyperthermia on EMS induced adaptive response in *in vivo* system of *P. pictus* and strengthened that there is high activity of repair mechanisms.

Declaration of interest

The authors declare that they have no competing interests.

Acknowledgements

We wish to express our gratitude to the Chairman, Department of studies in Zoology, University of Mysore and Applied Zoology, Kuvempu University, Shivamogga for providing facilities and to the University Grants Commission, India for awarding funding for the project (F.3-58/93SR-II).

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