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# Hyperthermia enhances methyl methanesulfonate-induced adaptive response in meiotic cells of grasshopper *Poecilocerus pictus*

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## Abstract

To understand the role of hyperthermia (HT) in adaptive response, methyl methanesulfonate (MMS) adapted meiotic cells of *Poecilocerus pictus* were used. *Poecilocerus pictus* were treated with conditioning (L) or challenging (H) dose of MMS and 2-h time lag (TL) between these doses (L-2h-H) (combined) was employed. Different treatment schedules were used to analyse the influence of HT on MMS-induced adaptive response namely pre; inter; post-treatment and cross-adaptation. After each treatment schedules, chromosomal anomalies were analysed. The frequencies of chromosomal anomalies induced by conditioning and challenging doses of MMS were significantly higher ($P < 0.0001$) compared to that of the control or HT groups. The combined treatments resulted in significant reduction of chromosomal anomalies compared to additive effect of MMS ($P < 0.0001$). The pre, inter, post and cross-adaptation treatments with HT reduced the frequencies of chromosomal anomalies compared to the challenge and combined treatments with MMS. There is a protection against MMS-induced chromosomal anomalies by HT in *in vivo P.pictus*. This is the first report to demonstrate that HT enhances the MMS-induced adaptive response in *in vivo* meiotic cells.

## Introduction

Hyperthermia (HT) (thermotherapy) is a type of cancer treatment in which body tissue is exposed to high temperatures (41–45°C) to damage and kill cancer cells or impede their further growth. It is a non-invasive cancer therapeutic tool which is being employed along with traditional radiotherapy, chemotherapy or combination of both (1). Many clinical trials have studied HT in combination with radiation therapy and/or chemotherapy that showed a significant reduction in tumour size by enhancing the effects of anticancer drugs or radiations and also reduce doses of anticancer drugs and radiations administered to patients (2). Reduction in doses helps to lessen the side effects of anticancer therapy (3). Hyperthermia is a weak mutagen but not shown to be a carcinogen (4); acts as synergistic agent with radiation and anticancerous drugs making it a potent adjuvant treatment in cancer therapy. The exposure of cells to temperature ranges for short periods of time, from 10 to 30 min allowing the development of tolerance towards subsequent exposure; this phenomenon is termed thermotolerance (5). On the other hand, phenomenon of adaptive response is a relatively small 'conditioning' dose induced less effective when the cells are treated with 'challenging' (higher) doses of same agent several hours later (6). This was first reported in plants (7) and later in bacteria (6). Its induction and manifestation have been studied in many organisms including *in vitro* human cells (8). Adaptive response has been assessed using multiple cytogenetic biomarkers-chromosome aberrations (CA), micronuclei (MN), sister chromatid exchanges (SCE) etc (9). Adaptive response is an important general biological mechanism to maintain genetic integrity of an organism. 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 cytotoxicity and mutagenesis (10). Contrary, HT can induce adaptation to cytogenetic damages caused by subsequent mutagenic agents (9,11). It is clear from the published data that there are contradictory reports about the action of HT and induction of adaptive response by HT in combination with alkylating agents.

Methyl methanesulfonate (MMS) has been used as a solvent, insecticide and chemotherapeutic alkylating agent. Human exposure to MMS is common in daily life through occupational sources, medical treatments and several endogenous sources that alkylate DNA. This DNA reactive chemical has cytotoxic and mutagenic properties, capable of inducing a variety of lesions including DNA adducts, cross-links and strand breaks, which can be expressed as chromosomal aberrations (12). In spite of extensive usages of this alkylating agent for cancer therapy, there are no reports on this agent to induce adaptive response with respect to HT administration. Understanding of this is utmost important in these days of triple modality of cancer therapy. In the present investigations, an attempt has been made to understand the influence of HT on MMS-induced adaptive response in meiotic cells of the grasshopper.

## Materials and methods

### Chemical

The monofunctional alkylating agent, MMS (CAS No. 66-27.3), a methylating agent obtained from Sigma Chemical Company was used.

### *Poecilocerus pictus*

Male grasshoppers weighing 2.5–3.0 g were collected from the environs of Mysore city and these were maintained in the laboratory for 2–3 days until use.

### Selection of chemical doses

In order to understand the adaptive response, the conditioning (0.012 M) and challenging (0.048 M) doses of MMS which were established already in previous experiments with *P.pictus* (13), were employed.

### Hyperthermia

Pilot toxicity studies were carried out to select the higher temperatures (HT) and the time of exposure. The grasshoppers were placed in the small cages and hyperthermic exposure was carried out using biological oxygen demand (BOD) incubator. Initial experiments were carried out by subjecting grasshoppers to temperatures from 38 to 45°C with 15 or 30 min exposure. The higher temperatures of 40 and 45°C with exposure time of 30 min were selected in the present study. The effective hyper temperatures were chosen based on the mortality and frequency of chromosomal anomalies produced in the pilot experimental studies.

### Treatment schedules

MMS was dissolved in 0.4% NaCl solution. Fifty microlitres of the fixed concentration of the chemical agent was injected into the abdomen of the animal between third and fourth segments. Each time freshly prepared solution of agent was used.

**Control**

The grasshoppers received 50 µl of 0.4% NaCl solution only.

**Hyperthermia (HT)**

The grasshoppers were exposed to 40 or 45°C for 30 min.

MMS treatment: The grasshoppers were treated with conditioning (L) or challenging (H) dose of MMS.

**Combined treatment of MMS**

The previous study (13) has shown that the combined treatment of conditioning and challenging doses of MMS with 2-h time lag (TL) between them offered appreciable protection in meiotic cells of *P.pictus*. In these experiments, the same 2-h TL between conditioning and challenging doses of MMS (L-2h-H) was employed to understand the occurrence of adaptive response.

Pre-treatments of HT: Grasshoppers were subjected to HT 2 or 4 h prior to conditioning dose of MMS and then they were challenged with same clastogen after 2 h.

1. [HT [40°C-30 min]-2h-L-2h-H]
2. [HT [45°C-30 min]-2h-L-2h-H]
3. [HT [40°C-30 min]-4h-L-2h-H]
4. [HT [45°C-30 min]-4h-L-2h-H]

**Inter-treatments of HT**

The grasshoppers were subjected to HT in between the conditioning and challenging treatment of MMS. Grasshoppers were exposed to HT for 1 h after conditioning dose of MMS and 1 h later they were challenged with challenging dose of the same clastogen with 30 min of HT.

1. [L-1h-HT [40°C-30 min]-1h-H]
2. [L-1h-HT [45°C-30 min]-1h-H]

**Post-treatments of HT**

Grasshoppers were exposed to HT, 2 or 4 h after combined treatment (L-2h-H) of MMS.

1. L-2h-H-2h-HT [40°C-30 min]
2. L-2h-H-2h-HT [45°C-30 min]
3. L-2h-H-4h-HT [40°C-30 min]
4. L-2h-H-4h-HT [45°C-30 min]

**Cross adaptation**

Grasshoppers were exposed to HT first and then the same animals were challenged after 2 h with challenging dose of MMS.

1. [HT [40 °C-30 min]-2h-H]
2. [HT [45 °C-30 min]-2h-H]

All the treated and control animals were maintained on fresh *Calatropis* leaves in the respective cages. The grasshoppers were sacrificed at 12, 24, 36 or 48 h of recovery times (RTs). Three experiments were carried out and a total of 12 animals were used for each treatment schedule.

### Meiotic chromosome preparation

Chromosome preparations were made following the procedure of Riaz Mahmood and Vasudev (14). The male grasshopper from the respective experimental treatment schedule was anesthetised by using chloroform and then dissected in insect saline. The testes lie in a dorsal position in the anterior half of the abdomen was easily located by making a dorsal, longitudinal and abdominal cut. They were identified by the orange-yellow fatty tissue that covers them. Each testis consists of many follicles like bunch of grapes. These testes were removed with dissecting needles, transferred to a clean jar

containing Carnoy's fixative (1:3-acetic acid: ethyl alcohol). The testis material was left in the fixative until it turns colourless; the old fixative was replaced with fresh Carnoy's fixative 2 or 3 times. The dehydration of testis material was performed by downgrading the colourless testis lobes in 100, 90, 80 and 70% of alcohol for 10 min each. Then testis material was stored in 70% ethyl alcohol until it was used for staining and chromosome slide preparation. Testes preserved in 70% alcohol was washed in distilled water thoroughly and then treated with 4% iron alum mordent for 20 min. The mordent was removed after the required period, followed by washing in water, 0.5% Heidenhein's hematoxylin stain was added to the mordented testes for 20 min. This step is very important because without the use of mordent hematoxylin stain solution is entirely ineffective in staining chromosome. The tubules were turned into black colour. One or two tubules of these were transferred on to grease-free clean slide containing a drop of 45% acetic acid. The material was minced well with sharp blade so has to get fine suspension of material. Then coverslip was placed and pressed hard using filter paper; sealed with paraffin wax. This is temporary squash preparation of testes chromosome.

### Chromosome analysis

Stained coded slides from grasshoppers belong to various treatment schedules 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 (Figures 1–5). Data was pooled from three independent experiments; minimum of 500 cells in each meiotic stage per dose were scored.

### Statistical analysis

The frequency of chromosomal anomalies scored in the different treatment schedules presented in the mean ± SE. The difference that exists among the treatment groups was analysed using the Tukey post hoc test using the SPSS software (version 16.0).

## Results

The observed frequencies of different types of chromosomal anomalies such as stickiness, stickiness and clumping, fragments, bridges, pseudobridges and laggards of 12-h RT are given in Table 1. Stickiness, stickiness and clumping were prominent in MMS treatment compared to controls and HT. At different RTs, both conditioning and challenging doses of MMS-induced significant chromosomal anomalies compared to control and HT (40 and 45°C) exposed for 30 min duration ($P < 0.0001$). Significant chromosomal anomalies were recorded at 12-h RT compared to 48-h RT ($P < 0.0001$). Combined treatment with 2-h TL between them resulted in significant reduction of chromosomal anomalies compared to that of additive effect at different RTs (Table 2).

Pre- treatment of HT at different treatment schedules to MMS-exposed cells resulted in significant reduction of chromosomal anomalies compared to that of combined treatment (L-2h-H) ($P < 0.0001$). The reduction of chromosomal anomalies is also evident in different temperatures, RTs and time intervals (Table 3). The frequencies of chromosomal anomalies were significantly reduced when HT was given between conditioning and challenging doses of MMS at all RTs ($P < 0.0001$). The percentage reduction of chromosomal anomalies observed is 20.90 to 37.01% compared to that of combined treatment (L-2h-H) (Table 4). There is a significant decrease in chromosomal anomalies in post-treatment of HT compared to combined treatment of MMS ($P < 0.001$). The percentage reduction of chromosomal anomalies is 1.50–13.58% compared to combined treatment of MMS (Table 5). The treatment of HT prior to challenging dose (i.e. HT + challenging dose) reduced chromosomal anomalies significantly compared to challenging dose at all RTs ($P < 0.0001$). The reduced yield of chromosomal anomalies is 23.33 to 26.95% at different temperatures and RTs (Table 6). The reduction of chromosomal anomalies were quite different at different temperatures and time intervals, more reduction of chromosomal anomalies were recorded 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 4-h time interval noticed high reduction

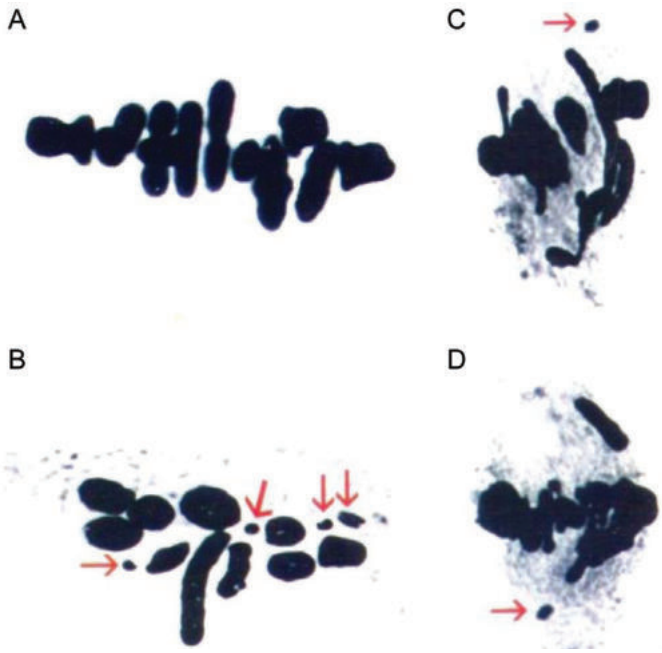


**Fig. 1.** Influence of MMS on metaphase I of meiotic cells of *P.pictus*. (**A**) Normal chromosome complement. (**B**) Chromosome fragments (→). (**C, D**) Chromosome stickiness, clumping and fragment (→).

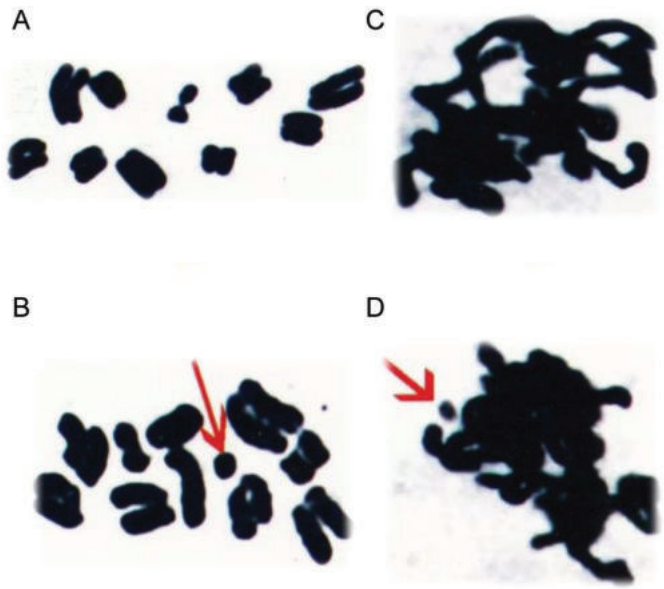


**Fig. 2.** Influence of MMS on Metaphase II of meiotic cells of *P.pictus*. (A) Normal chromosome complement. (B) Chromosome fragment (→). (**C**) Chromosome stickiness and clumping (→). (**D**) Chromosome stickiness and fragment (→).

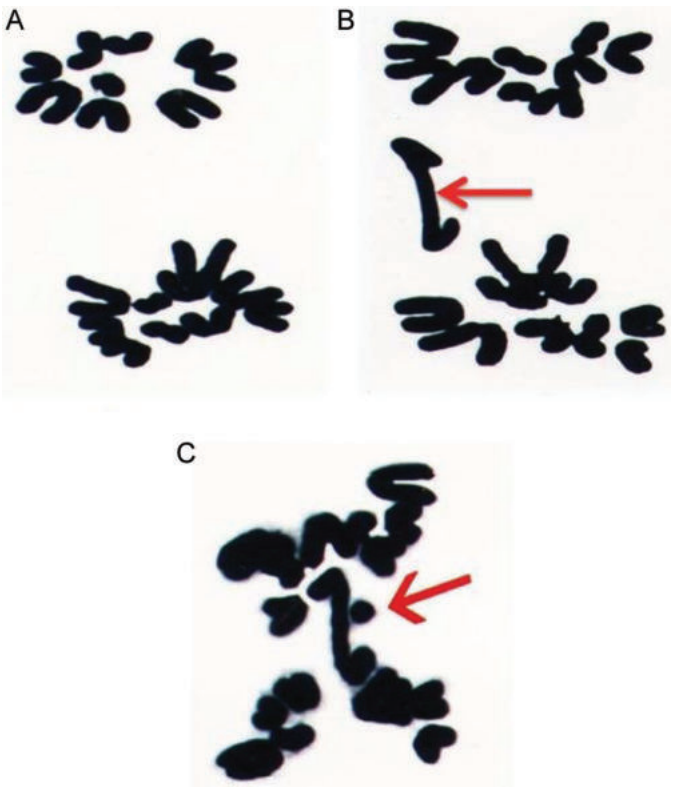


**Fig. 3.** Influence of MMS on Anaphase I of meiotic cells of *P.pictus*. (**A**) Normal chromosome complement. (**B**) Chromosome bridge (→). (**C**) Chromosome bridge and fragment (→).

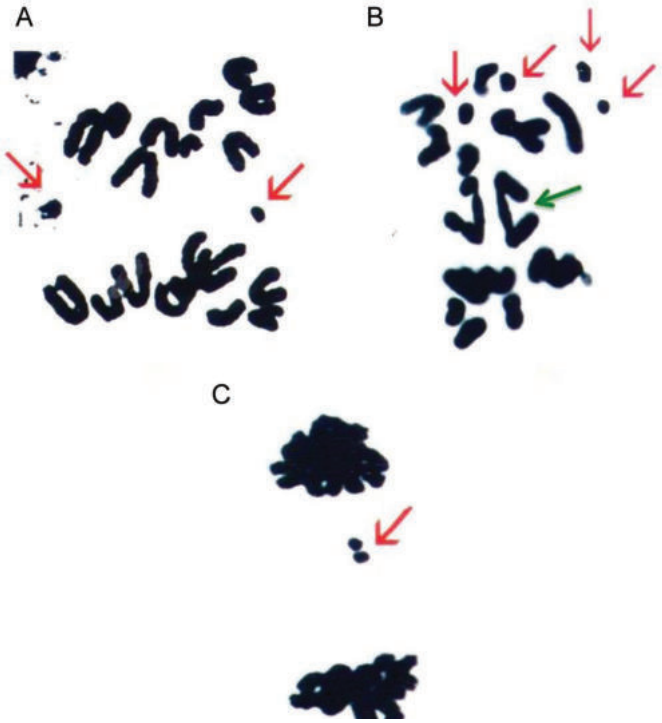


**Fig. 4.** Influence of MMS on Anaphase I of meiotic cells of *P.pictus*. (**A**) Chromosome laggards (→). (**B**) Chromosome bridges (→). Chromosome fragments (→). (**C**) Chromosome laggards (→).

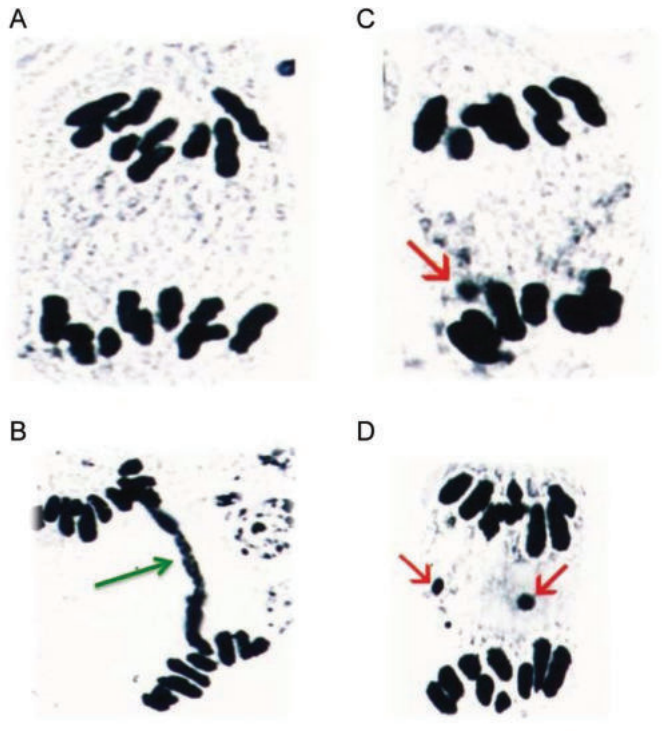


**Fig. 5.** Influence of MMS on anaphase II of meiotic cells of *P.pictus*. (**A**) Normal chromosome complement. (**B**) Chromosome bridge (→). (**C**) Chromosome fragment (→). (**D**) Chromosome laggard (→).

**Table 1.** Frequency (%) of individual chromosomal anomalies (mean ± SE) observed after hyperthermia (HT) or MMS treatment in meiotic cells of *P.pictus* at 12-h recovery time (RT)

| 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 |
| HT [40°C–30 min] | 1.38 ± 0.037 | — | — | 0.10 ± 0.048 | 1.44 ± 0.070 | — | 3.68 ± 0.218 | 0.42 ± 0.056 | — | — | — | — | 7.02 ± 0.423 |
| HT [45°C–30 min] | 1.48 ± 0.031 | — | — | 0.11 ± 0.018 | 1.47 ± 0.038 | — | 3.57 ± 0.030 | 0.44 ± 0.020 | — | — | — | — | 7.07 ± 0.404 |
| MMS-L | 3.58 ± 0.019 | 2.26 ± 0.017 | 1.02 ± 0.006 | 1.02 ± 0.006 | 0.82 ± 0.013 | 0.01 ± 0.006 | 16.07 ± 0.057 | 13.01 ± 0.043 | 0.59 ± 0.022 | 0.97 ± 0.008 | 0.85 ± 0.018 | 0.01 ± 0.003 | 40.21 ± 0.175[a] |
| MMS-H | 15.30 ± 0.018 | 7.44 ± 0.022 | 1.56 ± 0.006 | 3.41 ± 0.024 | 5.71 ± 0.027 | 0.29 ± 0.010 | 35.60 ± 0.049 | 16.74 ± 0.037 | 2.69 ± 0.026 | 4.21 ± 0.012 | 1.12 ± 0.012 | 0.42 ± 0.057 | 94.49 ± 0.146[a] |
| L-2h-H | 15.46 ± 0.035 | 5.66 ± 0.023 | 1.38 ± 0.036 | 1.58 ± 0.019 | 4.59 ± 0.027 | 0.30 ± 0.035 | 21.36 ± 0.012 | 16.86 ± 0.016 | 3.52 ± 0.018 | 2.92 ± 0.025 | 2.55 ± 0.049 | 0.13 ± 0.009 | 76.31 ± 0.129[a] |

Pooled data from three independent experiments; minimum of 500 cells in each meiotic stage per dose were scored; four grasshoppers per experiment were used. Br, bridges; Fr, fragments; H, challenging dose; HT, hyperthermic treatment; Lag, Laggards; L, conditioning dose; min, minutes; PB, pseudo bridges; St, Stickiness; St&Cl, stickiness and clumping.

[a]Significant against to control and hyperthermic treatment at $P < 0.0001$ according to Tukey post hoc test.

of chromosomal anomalies than at 2-h time interval at all RTs (Tables 3–6).

## Discussion

Grasshopper (*P.pictus*) is a monophagous pest on calotropis plant, easy to handle and economical to maintain in laboratory conditions. Grasshoppers are classic experimental material for cytologist for demonstrating cytology-related research work. The chromosomes of the grasshoppers have been used for a vast number of cytological studies. The chromosomes are large and relatively few in number—male: $2n$ = 19 (X0); female: $2n$ = 20 (XX). The range of chromosome lengths in the complement is such that each bivalent formed at meiosis can usually be individually identified according to its length. Chiasmata are very clear during diplotene thus allowing analyses of their structure, frequency, distribution and movement. Often the position of the centromere is marked by relatively denser staining (condensation) at early diplotene. The techniques involved in the preparation of slides of this material are quick and simple; therefore it is ideal for demonstrating the stages of meiosis. The number of cells available for scoring chromosomal anomalies is high. The abundance of meiotic cells in all stages is an asset of grasshoppers over mammalian test systems. The effect of any physical or chemical agents can be detected in larger detail than in any other test systems. Several reports are available on the clastogenic effects of physical and chemical agents on the chromosomes of *P.pictus* (12–17). The natural populations of this species occur in their habitat as isolated pockets and hence the degree of inbreeding is very high, almost resembling an isogenic state if not exactly achieving it like laboratory breed animals. Hence, *P.pictus* has been employed as a sub mammalian *in vivo* system to analyze the influence of HT on MMS-induced adaptive response.

Cytogenetic studies with grasshopper showed the induction of chromosome breakage by alkylating agents (9,13–16). MMS is a methylating agent, inducing different types of meiotic chromosomal anomalies in *P.pictus viz.*, stickiness, stickiness and clumping, fragments, bridges, laggards and pseudo bridges (18). The chromosome stickiness and stickiness and clumping are the prominent ones with high frequencies of recorded chromosomal anomalies (Table 1). Stickiness has been reported to be induced by alkylating agents in *P.pictus* (9,15). Stickiness resulted from the breakdown of chromosomal nucleic acid into the depolymerised fluid state, the dissociation of nucleic acid into the nuclear sap, high proteolytic activity and excess histone might have cross-link DNA in the neighbouring strands (18,19). In stickiness and clumping, chromosome complement stuck together, formed irregular masses and in the extreme chromosome clump, individuality of chromosome is lost. The electron microscopic study reported that mammalian sticky chromosomes and *Allium cepa* root tip induced by chemicals possess fine fibrous connections between chromosomes and these are chromatid fibres (20). From this, it can be considered that chromosome stickiness is a chromatid type of aberration.

The genetic effects of toxic agents, when produced in germ cells, lead to an increase of the genetic load of our descendants (21). These genetic effects can be rectified by nature gifted error correcting mechanisms called genetic repair mechanisms or DNA repair pathways by which the organisms combat the effects of toxic agents. Many defense mechanisms have evolved to minimise genotoxic damages and these are conserved in diverse groups of organisms such as bacteria, yeast, insect, mammals and plants including humans (22). Error-free inducible DNA repair known as adaptive response (6) is well-documented.

**Table 2.** Percentage of chromosomal anomalies (mean ± SE) observed after HT or MMS treatment in meiotic cells of *P.pictus* at different RTs

| Treatment groups | % Chromosomal anomalies at different RTs (h) | | | |
|---|---|---|---|---|
| | 12 | 24 | 36 | 48 |
| Control | 7.09 ± 0.249 | 7.10 ± 0.126 | 7.12 ± 0.071 | 7.17 ± 0.096 |
| HT [40 °C–30 min] | 7.02 ± 0.433 | 7.07 ± 0.394 | 7.07 ± 0.403 | 7.15 ± 0.289 |
| HT [45°C–30 min] | 7.07 ± 0.404 | 7.12 ± 0.556 | 7.14 ± 0.510 | 7.17 ± 0.470 |
| MMS-L | 40.77 ± 0.885[a] | 39.02 ± 0.985[a] | 38.11 ± 0.650[a] | 37.17 ± 0.585[a] |
| MMS-H | 94.49 ± 0.646[a] | 89.07 ± 0.754[a] | 86.10 ± 0.743[a] | 83.05 ± 0.722[a] |
| L-2h-H | 76.31 ± 0.729[a] | 73.24 ± 0.903[a] | 70.07 ± 0.798[a] | 67.03 ± 0.823[a] |
| % Reduction | 43.58 ± 0.980 | 42.82 ± 0.878 | 43.59 ± 0.683 | 44.24 ± 0.652 |

Pooled data from three independent experiments; minimum of 500 cells in each meiotic stage per dose were scored; four grasshoppers per experiment were used. Individual chromosomal anomalies were scored as per Table 1 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-2h-H); % of reduction in chromosomal anomalies (C) was calculated by using formula: C = (B/A × 100) – 100.

[a]Significant against to control and HT at $P < 0.0001$ according to Tukey post hoc test.

**Table 3.** Percentage of chromosomal anomalies (mean ± SE) observed after pretreatment of HT to combined (conditioning and challenging) doses of MMS-treated meiotic cells of *P.pictus* at different RTs

| Treatment groups | % Chromosomal anomalies at different RTs (h) | | | |
|---|---|---|---|---|
| | 12 | 24 | 36 | 48 |
| L-2h-H | 76.31 ± 0.729 | 73.24 ± 0.903 | 70.07 ± 0.798 | 67.03 ± 0.823 |
| HT [40°C–30 min]-2h-L-2h-H | 64.18 ± 0.613[a] (18.89) | 59.41 ± 0.517[a] (23.28) | 55.36 ± 0.733[a] (26.56) | 52.50 ± 0.278[a] (27.67) |
| HT [45°C–30 min]-2h-L-2h-H | 62.17 ± 0.335[a] (22.73) | 57.17 ± 0.976[a] (28.11) | 54.05 ± 0.562[a] (29.63) | 50.30 ± 0.426[a] (33.25) |
| HT [40°C–30 min]-4h-L-2h-H | 60.07 ± 0.727[a] (27.02) | 55.49 ± 0.573[a] (31.99) | 53.13 ± 0.326[a] (31.87) | 47.46 ± 0.106[a] (41.24) |
| HT [45°C–30 min]-4h-L-2h-H | 58.09 ± 0.436[a] (31.36) | 53.65 ± 0.415[a] (36.50) | 53.23 ± 0.427[a] (31.63) | 46.83 ± 0.118[a] (43.13) |

Pooled data from three independent experiments; minimum of 500 cells in each meiotic stage per dose were scored; four grasshoppers per experiment were used. Individual chromosomal anomalies were scored as per Table 1 and pooled to make data concise, thus the percentage anomalies for different recovery times are given in this table. Values in parenthesis are percentage reduction of chromosomal anomalies compared to combined treatment (L-2h-H). Calculation of percent reduction: (A) Chromosomal anomalies observed in respective pre HT (HT-2h-L-2h-H); (B) Chromosomal anomalies observed in combined treatment (L-2h-H); % of reduction in chromosomal anomalies (C) was calculated by using formula: C = (B/A × 100) – 100.

[a]Significant against to combined treatment (L-2h-H) at $P < 0.0001$ according to Tukey post hoc test.

**Table 4.** Percentage of chromosomal anomalies (mean ± SE) observed after inter treatment of HT between conditioning and challenging doses of MMS in meiotic cells of *P.pictus* at different RTs

| Treatment groups | % Chromosomal anomalies at different RTs (h) | | | |
|---|---|---|---|---|
| | 12 | 24 | 36 | 48 |
| L-2h-H | 76.31 ± 0.729 | 73.24 ± 0.903 | 70.07 ± 0.798 | 67.03 ± 0.823 |
| L-1h-[HT-40°C–30 min]-1h-H | 63.11 ± 0.374[a] (20.90) | 58.98 ± 0.504[a] (24.17) | 55.09 ± 0.343[a] (27.20) | 52.33 ± 0.159[a] (28.10) |
| L-1h-[HT-45°C–30 min]-1h-H | 60.07 ± 0.357[a] (27.04) | 54.48 ± 0.365[a] (34.43) | 51.33 ± 0.494[a] (36.51) | 48.92 ± 0.563[a] (37.01) |

Pooled data from three independent experiments; minimum of 500 cells in each meiotic stage per dose were scored; four grasshoppers per experiment were used. Individual chromosomal anomalies were scored as per Table 1 and pooled to make data concise, thus the percentage anomalies for different recovery times are given in this table. Values in parenthesis are percentage reduction of chromosomal anomalies compared to combined treatment (L-2h-H). Calculation of percent reduction: (A) Chromosomal anomalies observed in respective inter HT (L-1h-HT-1h-H); (B) Chromosomal anomalies observed in combined treatment (L-2h-H); % of reduction in chromosomal anomalies (C) was calculated by using formula: C = (B/A × 100) – 100.

[a]Significant against to combined treatment (L-2h-H) at $P < 0.0001$ according to Tukey post hoc test.

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 MMS in meiotic cells of *P.pictus* (Table 2). Similar results have been recorded in the induction of adaptive response in *Vicia faba*, *P.pictus*, *Drosophila*, mouse and human lymphocytes by alkylating

**Table 5.** Percentage of chromosomal anomalies (mean ± SE) observed after post-treatment of HT to combined (conditioning and challenging) MMS dosed in meiotic cells of *P.pictus* at different RTs

| Treatment groups | % Chromosomal anomalies at different RTs (h) | | | |
|---|---|---|---|---|
| | 12 | 24 | 36 | 48 |
| L-2h-H | 76.31 ± 0.729 | 73.24 ± 0.903 | 70.07 ± 0.798 | 67.03 ± 0.823 |
| L-2h-H-2h [HT-40°C–30 min] | 74.49 ± 0.614[a] (2.44) | 72.16 ± 0.405[a] (1.50) | 68.81 ± 0.784[a] (1.83) | 64.81 ± 0.281[a] (3.44) |
| L-2h-H-2h [HT-45°C–30 min] | 73.10 ± 0.724[a] (4.38) | 71.13 ± 0.775[a] (2.96) | 66.98 ± 0.338[a] (4.61) | 62.51 ± 0.534[a] (7.23) |
| L-2h-H-4h [HT-40°C–30 min] | 71.22 ± 0.706[a] (7.14) | 70.09 ± 0.563[a] (4.49) | 68.98 ± 0.456[a] (1.58) | 60.12 ± 0.874[a] (11.50) |
| L-2h-H-4h [HT-45°C–30 min] | 69.04 ± 0.883[a] (10.53) | 68.97 ± 0.784[a] (6.18) | 66.91 ± 0.634[a] (4.72) | 59.01 ± 0.346[a] (13.58) |

Pooled data from three independent experiments; minimum of 500 cells in each meiotic stage per dose were scored; four grasshoppers per experiment were used. Individual chromosomal anomalies were scored as per Table 1 and pooled to make data concise, thus the percentage anomalies for different recovery times are given in this table. Values in parenthesis are percentage reduction of chromosomal anomalies compared to combined treatment (L-2h-H). Calculation of percent reduction: (A) Chromosomal anomalies observed in respective post HT (L-2h-H-HT); (B) Chromosomal anomalies observed in combined treatment (L-2h-H); % of reduction in chromosomal anomalies (C) was calculated by using formula: C = (B/A × 100) – 100.

[a]Significant against to combined treatment (L-2h-H) at $P < 0.0001$ according to Tukey post hoc test.

**Table 6.** Percentage of chromosomal anomalies (mean ± SE) observed in meiotic cells of *P.pictus* treated with HT and challenging with high dose of MMS at different RTs

| Treatment groups | % Chromosomal anomalies at different RTs (h) | | | |
|---|---|---|---|---|
| | 12 | 24 | 36 | 48 |
| MMS-H | 94.49 ± 0.646 | 89.07 ± 0.754 | 86.10 ± 0.743 | 83.05 ± 0.722 |
| [HT-40°C–30 min]-2h-H | 71.14 ± 0.386[a] (24.71) | 68.28 ± 0.614[a] (23.33) | 65.45 ± 0.459[a] (23.97) | 62.02 ± 0.537[a] (25.33) |
| [HT-45°C–30 min]-2h-H | 69.03 ± 0.837[a] (26.95) | 67.13 ± 0. 540[a] (24.63) | 63.61 ± 0.574[a] (26.11) | 61.18 ± 0.615[a] (26.34) |

Pooled data from three independent experiments; minimum of 500 cells in each meiotic stage per dose were scored; four grasshoppers per experiment were used. Individual chromosomal anomalies were scored as per Table 1 and pooled to make data concise, thus the percentage anomalies for different recovery times are given in this table. Values in parenthesis are percentage reduction of chromosomal anomalies compared to challenging dose of MMS. Calculation of percent reduction: (A) Chromosomal anomalies observed in challenging dose (H); (B) Chromosomal anomalies observed in respective HT (HT-2h-H); % of reduction in chromosomal anomalies (C) was calculated by using formula: C = (B/A × 100) – 100.

[a]Significant against to challenging dose at $P < 0.0001$ according to Tukey post hoc test.

agents (9,15,23,24). These findings indicate the presence of inducible protective repair mechanisms. Although the adaptive response system in bacteria is well validated, the existence of such a mechanism in eukaryotic cells is not yet established. Furthermore, underlying mechanisms of clastogenic adaptation in eukaryotic *in vivo* systems are presently unknown that too in meiotic cells; in this direction an attempt has been made in the present study.

The HT is a weak mutagen and not carcinogen (4) but it is also not inducing significant chromosomal anomalies (25). Contrary to these, it has been demonstrated that HT could induce chromosomal aberrations in *in vitro* Chinese hamster ovary (CHO) cells, He La cells and human A549 cells (26). There are conflicting reports on the effects of HT on chromosome and there are no sufficient reports on the effects of HT using *in vivo* systems. Hyperthermia of 40°C or 45°C exposed for 30 min could not induce significant anomalies in meiotic cells of *P.pictus* at all RTs analyzed compared to that of control ($P > 0.05$; Tables 1 and 2). This is the first report in meiotic cells of *in vivo* system *P.pictus*.

The significant decrease of chromosomal anomalies in meiotic cells of *P.pictus* ($P < 0.0001$; Table 3), after pre-treatment of HT 2 or 4 h prior to combined treatment demonstrate the enhancement of adaptive response by HT in *in vivo* system. Similarly, when HT as adaptive dose was given and then challenged with an interval of 6 h, there was a reduction in the number of chromatid and isochromatid breaks ranging from 30 to 70% in human lymphocytes (11). The present study demonstrated that when HT was given as first adaptive dose and conditioning dose as second adaptive dose, then there was also highly significant induction of adaptation to subsequent challenge dose of the MMS in *P.pictus*. For example 23.28 to 36.50% reduction of chromosomal anomalies in treatments of HT + adaptive dose of MMS + high dose of MMS in meiotic cells of *P.pictus* at 24 h RT compared to combined doses of MMS (L-2h-H) (Table 3). These results suggest that there is an additive or synergistic effect proving that the adaptation induced by HT involves the different mechanisms compared to chemical adaptation. Rieger and Michaelis (27) have shown reduction in maleic hydrazide (MH) or triethylenemelamine (TEM) induced chromatid aberrations in the cells which were pre-exposed to 40°C HT for 10 min. They also demonstrated that the protective function of HT is a quick response, which lasts up to 4 h, suggesting that HT before clastogen treatment triggers clastogen-specific protective functions, which eventually result in protection against clastogens. Similarly, there was a reduction in the chromatid aberrations in *V. faba* seedlings which were pre-treated

with HT of 40°C for 10 min and then challenged with *N*-methyl-*N*-nitrosourea (MNU) when compared with challenging treatment of MNU alone (28). These evidences indicate the beneficial role of conditioning treatment of HT in reducing genetic damages.

The inter-treatment of HT (L-1h-HT-1h-H) with MMS yielded significantly less frequency of chromosomal anomalies compared to combined treatment (L-2h-H) indicating the enhancement of adaptive response by HT in *P.pictus* ($P < 0.0001$; Table 4). When human lymphocytes were subjected to HT in between the conditioning and challenging treatment of X rays, reduced the chromatid and isochromatid breaks of the effects induced by challenge dose alone (11). Bleomycin (BLM) given intra peritoneal before HT and then radiation was administered as five fractions of 3 Gy resulted in increased growth delay upto 14.5 days in FSaIIC fibrosarcoma tumour cells (29). Similar type of reduction in chromosomal anomalies were recorded when HT was given between conditioning and challenging doses of ethylating agent, ethyl methane sulfonate (EMS) at all RTs (9). As it has been discussed in the pre-treatments even inter treatment of HT showed clasto-resistance irrespective of temperatures (Table 4).

In the post-treatments of HT after 2 or 4 h time interval in *P.pictus* yielded significantly lower frequencies of chromosomal anomalies compared to combined treatments at all RTs tested ($P < 0.0001$; Table 5). Hyperthermia as a post-treatment after the administration of BLM followed by radiation produced 1.5 to 2.5-fold greater tumour cell killing than radiation-BLM-HT in FSaIIC fibrosarcoma tumour cell line (29). Contrary to present finding, post-treatments of HT-treated cells with Trenimone (trifunctional alkylating agent) has 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 (30). There was a decrease in chromosomal anomalies in post-treatment of HT compared to combined treatment of EMS (9). In the present study, the % reduction of chromosomal anomalies is between 1.50 and 13.58% (Table 5) in post-treatment of HT compared to combined treatment of MMS. Generally, methylating agents are ~10 to 20-fold more reactive than ethylating agents, as the larger groups are less efficiently transferred. Ethyl adducts are considered more mutagenic, probably as methyl adducts are repaired with greater efficiency (31). However, factors, such as alkylating agent reactivity and the sequences they preferentially target, must also be taken into account. The present results show that adaptive dose + challenge dose along with HT of different temperatures and time intervals can induce the adaptation to cytogenetic damages in *P.pictus*. Percentage reduction in post-treatment of HT was not significant compared to pre and inter treatment this may be because of the fact that HT may not act as precursor for already established adaptive response by low dose chemical agent.

In this study, when *P.pictus* was exposed to HT first and then the same animals were challenged with high dose of MMS yielded significantly reduced chromosomal anomalies compared to that of combined treatment ($P < 0.0001$; Table 6). This suggests that there is a cross-adaptation in meiotic cells. An adaptive response to HT of this type was first observed in *E. coli* (32) and then in human lymphocytes (33). When CHO cells preheated at 43°C before drug exposure reduced the cytotoxicity of actinomycin D (34). The EMT6 mouse tumour cells were preheated for 3 h at 40°C along with cytotoxic agents such as BLM and 1,3-bis(2-chlorethyl) nitrosourea (BCNU) found to reduce cell killing and produced measurable protection (thermal tolerance) (35). Increase in the resistance of the cells to the chemotherapeutic agents such as adriamycin or actinomycin D, was noticed when prior HT was applied (36). The pre-treatment of CHO cells with the HT (46°C for 6 min) led to a reduction of Alu-I restriction endonuclease induced CAs (30). The chemotherapy has the greatest effect when administered with HT (36). The reduction in the frequencies of chromosmal anomalies in the hyperthermic treatment with 2 h before challenging dose reflect effects equivalent to conditioning dose of chemical agent (Table 6). Thus it can be opined that HT is acting like conditioning dose to induce adaptive response and it also an example of cross adaptation in meiotic chromosomes of *P.pictus* which is a first report.

Each treatment schedule also showed that there is a significant less reduction of chromosomal anomalies at 40°C compared to 45°C in *P.pictus* at all RTs tested (Tables 3–6). Hyperthermia administered for 10 or 30 min, 1 or 2 h prior to MH or MNU resulted in a significant decrease in the percentage of metaphases with chromatid aberrations at different RTs tested (37). Similar results were obtained when TEM instead of MH was used; prolongation of time interval, i.e. 2 h instead of 1 h between HT and TEM resulted in chromosomal aberrations yield near to the control value. A shorter duration of HT (10 min) proved to be insufficient to lower the TEM effects over the different RTs tested (27,37). On par with this, in the present study, HT was administered for 30 min 2 h prior to MMS dose in *P.pictus*; resulted a significant decrease in chromosomal anomalies in *P.pictus* at all RTs tested (Tables 3–6). Therefore, HT administered prior to MMS treatment reduced the clastogenic activity of both the agents with same time span. Experiments with human lymphocytes showed that full adaptation to ionising radiation did not occur until 4–6 h after the treatment of adaptive dose (8).

In this study, in all the treatments, different RTs (fixed times) have been employed. If one RT was selected, then one would have argued that the reduced anomalies yields observed after different treatments are due to the effects of pretreatment of HT in the cell cycle. To exclude this argument in the present investigations, different RTs were selected to study the induction of protection in different cell populations in *P.pictus*. The frequency of chromosomal anomalies induced after a 48-h RT was less compared to 12 and 24 h RTs (Tables 3–6). This could be due to the inclusion of second and subsequent meiosis during scoring after the treatment. The reduction of chromosomal anomalies with increasing RTs in the current study reflects the mechanism of meiotic selection, where chromosomal anomaly bearing cells are eliminated, similar to mitotic selection as has been demonstrated in treated mitotic cells of different organisms (14,15). 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.

## Conclusion

The administration of HT could not induce significant chromosomal anomalies compared to that of control at different RTs in *P.pictus*. Pre or post-treatment of HT to combined (L-2h-H) doses of MMS or inter treatment of HT in between the conditioning and challenging dose of MMS have revealed that there is a significantly reduced chromosomal anomalies compared to combined (L-2h-H) doses of MMS at different RTs. Further, when HT was given as conditioning dose the frequency of chromosomal anomalies were also significantly reduced; this shows that there is a cross-adaptation in meiotic cells as that of mitotic cells. Thus, the present study demonstrates that there is enhanced influence of HT on MMS induced adaptive response in *in vivo* meiotic cells of *P.pictus*.

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