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# Optimizing chromosome preparation in common carp: Effects of colchicine incubation and hypotonic treatment timing on chromosomal quality

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Karyotyping is an analytical technique to examine chromosomal structure, characteristics, and cell division. In karyotyping, stained preparations are photographed to arrange chromosomes. Modern methods such as karyotyping, make it easier to identify specific chromosomal pairs, which advances our knowledge of the chromosomal causes of important hereditary diseases. While karyotyping variations can occur within and between species, each organism has a distinct karyotype characterized by the number and shape of its chromosomes. The present study examined the quality of chromosomes obtained from nine treatments, each with varying colchicine incubation time and hypotonic treatment duration in juveniles of common carp (*Cyprinus carpio*). The study involved varying colchicine exposure times (1, 3, and 5 h) at a concentration of 0.05%, along with hypotonic solution exposure durations (35, 45, and 60 min) applied to kidney tissue. The study found that at a colchicine concentration of 0.05% and incubation times of 3–5 h, the number of metaphase chromosomes and the quality of chromosomal spreads correlated positively with 45 min of KCl treatment, proving more effective than other treatment durations.

**Keywords:** *Cyprinus carpio*, Exposure time, Karyotyping, Metaphase chromosome spreads

The study's primary objective is to improve the techniques for preparing high-quality chromosome spreads in common carp (*Cyprinus carpio*) by evaluating the impact of varying colchicine (a chemical that helps to stop cells at the right stage for chromosome studies) incubation time and hypotonic (helps spread out the chromosomes) treatment duration on the quality and clarity of chromosomal spreads. This entails determining which combinations of hypotonic treatment duration and incubation time, as well as colchicine concentration and incubation time produce the best metaphase chromosome spreads, improving the precision of karyotyping for additional genetic and chromosomal research. Therefore, this study aims to standardize the chromosome preparation protocol in common carp juveniles. The study seeks to determine the best timing for getting clear, well-separated chromosomes by testing different combinations of these treatments. As a result, it will be easier to study the fish chromosomes in detail. Fish chromosomal research holds significant importance, as it aids in the conservation of fish populations, facilitates the understanding of species classification, and investigates evolutionary relationships[1,2]. The number, size, and form of fish chromosomes have provided valuable insights into studying chromosome modification[3]. As compared to other vertebrates, fish chromosomes are particularly difficult to analyze due to their small cells and the number of chromosomes that group together during metaphase[4,5] It is important to study karyotyping for different fish species since it helps understand genetic variation within a species and to distinguish between different species[6]. More subtle structural changes such as chromosomal deletions, duplications, translocations, or inversions might be found by closely examining karyotypes. In fact, karyotypes are being utilized more and more to identify hereditary diseases, congenital disabilities and even cancer[7].Numerous karyotyping methods have been developed to visualize fish chromosomes at various developmental stages, including tissue cultures[8], squashing techniques[9], and cell suspensions of the tissues undergoing mitosis[10,11]. Numerous methods have been refined to achieve widely distributed mitotic chromosomes in fish, with variations possible among species e.g., as observed by Karami *et al.*[12]. These often relate to the kinds of chemicals, their concentrations, and the duration of exposure. Even if it is simpler to prepare fish chromosomes from eggs and larvae for cytogenetic investigations involving particular fingerling tissues, using juvenile or adult fish may have some advantages over using complete larvae or eggs. When examining the offspring of intergeneric hybridization, crossings between distinct fish species from different genera karyotyping is very helpful[13]. It facilitates the

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alignment of their morphological features with their genetic characteristics, which is crucial in cases of chromosomal number differences (ploidy polymorphism)[14-16]. Due to their resemblance in early development, hybrid eggs and larvae can be difficult to link to specific ploidy levels because they show morphological characteristics that are the same. Chromosomes can be prepared from various tissues in post-larval fish. These include, the kidney[16,17], gills[18], scales[19], fins[20], and gonads[21]. Despite effective isolation of chromosomes from different tissues, different rates of mitotic cell division can affect the quantity and quality of chromosomes found[22]. The effectiveness of metaphase interaction with cytogenetic chemicals may depend on when "cellular suspension" occurs (before or after chemical treatments)[13]. However, no studies have optimized the best time to prepare cell suspension. Several techniques are used to study fish chromosomes, some of which have been improved and modified. As an example, pretreatment with colchicine and hypotonic solution causes the cells to expand and the chromosomes to separate[2,23]. In addition to being one of the most widely used species in aquaculture, common carp has also been studied for its physiological factors and for infectious diseases[24].As a result of its big size, it has been able to conduct basic research on immunological recognition and the creation and function of organs, which is not possible in small fish species[25]. Karyotyping is essential for understanding the genetics of common carp in order to identify desirable characteristics for breeding, create detailed genetic maps for research, and monitor evolutionary changes. Common carp have a high number of chromosomes, usually 100–104[26], which makes the process complex and presents various challenges for karyotyping. Accurate identification and pairing of chromosomes is made more difficult by their identical appearance and small size. Furthermore, there can be differences in the quality of chromosome preparations, which can affect the karyotypes' clarity and detail. To get accurate results, handling and preparation practices must be followed. It takes skill to interpret these karyotypes because it might be difficult to identify small variances and structural abnormalities. It is essential to select the appropriate concentration for spreads and incubation time to obtain clear and identifiable metaphase chromosomes[27]. If the concentration of colchicine is less, then there is a possibility that an insufficient amount will fail to arrest the target cells at metaphase stage[28], however excessive concentration or extended exposure may cause chromosomal condensation[29]. A significant and vital component in enhancing chromosomal spreads is hypotonic treatment. This process denatures proteins and aids in the elimination of lipids. When cell contents are spread out on slides, hypotonic treatment permits cell enlargement, which promotes cell breakdown and chromosomal dispersion[23]. According to Ida *et al*.[30], solution of potassium chloride had the best chromosomal spreads when compared to two other hypotonic solutions of distilled water and sodium citrate. Chourrout & Happe[31] reported that, at low temperature the chromosomal spreading was insufficient at 0.56% KCl for hypotonic treatment in rainbow trout. However, when the tests were carried out at room temperature, the same KCl concentration produced somewhat superior outcomes. Giemsa stain is most frequently used for staining slides. When Arcement & Rachlin[32] experimented with a variety of stains, such as aceto-orcein, aceto-cannine, and Giemsa (normal or buffered), they discovered that Giemsa standard produced the best results. The majority of the workers, however, suggested diluting Giemsa with phosphate buffer.

## Materials and Methods

### Sample collection

For the study, a total of about 45 specimens of common carp juveniles were collected from the hatchery of Directorate of Coldwater Fisheries Research (DCFR), Bhimtal, located at 29° 21′ 0″ N, 79° 34′ 0.12″ E, and an altitude of 1370 m above mean sea level (MSL), in the Shivalik Range of the Himalayas. Nine treatments were taken for the experiment with varying colchicine incubation duration and hypotonic treatment exposure (Table 1). Five fish were kept in nine different treatments and fifteen slides were prepared from each fish.

### Preparation of reagents[34]

0.05% Colchicine; Weighed 5 mg of colchicine and diluted it with 10 mL of DDW and rest was stored in the refrigerator. 0.56% KCl: Dissolved 0.56 g KCl in 100 ml DDW (to be used fresh). A Giemsa stain (stock) was prepared by dissolving 0.5 grams of Giemsa powder in 33 ml of glycerol, incubating at 60° C overnight in a water bath, cooling to room temperature, adding 33 ml of methanol, mixing properly, and filtering with Whatman filter No.1. Preparation of 4–6% Giemsa stain

was achieved by dilution of stock solution in phosphate buffer solution. In order to dissolve Giemsa 4%, 2 mL of the stock solution was dissolved in 48 mL of phosphate buffer. For Giemsa 5%, 2.5 mL of the stock solution were dissolved in 47.5 mL of phosphate buffer, and for Giemsa 6%, 3 mL were dissolved in 47 mL of phosphate buffer.

**Chromosome preparation**

Chromosome preparation for *Cyprinus carpio* (weighing between 30-50g) was performed using the methods decribed by [33,34] with some modifications. Five samples were used for each treatment. The standard concentration of colchicine of 0.05% was freshly prepared and was injected intramuscularly at 1mL/100g of body weight for 1, 3 and 5 h to depress the mitotic division at the metaphase stage (Table 1). The fish were anesthetized using clove oil and were dissected. The kidney was removed, homogenized and hypotonised simultaneously in 0.56% KCl for 35, 45 and 60 mint room temperature in a 15 mL centrifuge tube. Thereafter, the hypotonic action was stopped by adding 1-2 mL freshly prepared chilled carnoy′s fixative (methanol: acetic acid in 3:1 ratio) gradually and mixed it gently. The cell suspension was then centrifuge at 1,200-1,500 rpm for 10 min at room temperature (RT) to get cell pellet at the bottom. Supernatant was removed leaving approximately 2-3 mL of supernatant. Then added 6-8 mL freshly prepared chilled fixative slowly and kept the tube in refrigerator for 1-2 h for thorough fixation. Afterwards the centrifugation process and the steps of removal of supernatant were repeated three times at 1 h interval till clear transparent cell suspension was obtained. Then took small quantity of cell suspension in a pasture pipette and drop it onto grease free, pre-cleaned glass slide from 1.5-2.0 feet height. The slides were stained with 4-6% Giemsa in phosphate buffer (*p*H 6.8) for 15-20 min. washed the slides with distilled water and air-dry them.

***Screening of slides***

The slides were observed under the field-illuminated trinocular microscope (Olympus CKX-53) fitted with camera at 40× objective and screened for good metaphase chromosomal spreads.

**Statistical analysis**

Two-way ANOVA (IBM SPSS statistic, version 26) was used to analyze the data collected from the different treatments to evaluate the significant differences between each treatment and their interactions. Following the ANOVA tests, Duncan's multiple range tests were used to detect significant differences ($P<0.05$) between groups.

## Results and Discussion

In this study, the quality of chromosomal spreads in nine distinct treatments were assessed, to analyze the effect of different timings of hypotonic treatment and colchicines incubation duration in the quality of metaphasic chromosomal spreads (Table 2). The number of chromosomal spreads (Mean ± SD) among all nine treatments is presented in Table 3. The highest (Mean ±SD**)** number of chromosomal spreads among the treatment groups was recorded in treatment T8 at 61.4 ± 2.27and the lowest (Mean ±SD) number of chromosomal spreads were recorded in treatment T2 at 1.79± 1.48. Although treatment T1 showed no spread as the cells remained intact (Fig.1A). This is because the exposure of colchicines incubation and hypotonic treatment timings were not significantly correlated ($P>0.05$) for the cell to swell and burst for an effective chromosomal spread. The spreads in treatments T5 (Fig. 1B) were 43.13±1.99 in numbers and were optimal because a significant ($P<0.05$) positive correlation was recorded between incubation duration of colchicines and the hypotonic treatment exposure. The substantial exposure to hypotonic treatment in T6 (Fig.1C), resulted in aggregated and indistinct chromosomal spreads (45 ± 2.42). The

Table 1 — Experimental details

| Treatments | Colchicine concentration (%) | Colchicine concentration Incubation timings (h) | Hypotonic treatment timings (min) | No. of samples/treatment | No. of slides/treatment |
|---|---|---|---|---|---|
| T1 | 0.05 | 1 | 35 | 5 | 15 |
| T2 | 0.05 | 1 | 45 | 5 | 15 |
| T3 | 0.05 | 1 | 60 | 5 | 15 |
| T4 | 0.05 | 3 | 35 | 5 | 15 |
| T5 | 0.05 | 3 | 45 | 5 | 15 |
| T6 | 0.05 | 3 | 60 | 5 | 15 |
| T7 | 0.05 | 5 | 35 | 5 | 15 |
| T8 | 0.05 | 5 | 45 | 5 | 15 |
| T9 | 0.05 | 5 | 60 | 5 | 15 |

chromosomal aggregation and spreading were found to be strongly affected by both colchicine concentration and incubation period along with high exposure to hypotonic treatment (Fig. 1C). In T9, excessive hypotonic treatment led to cell death, in which a positive correlation was not seen between colchicine incubation exposure and hypotonic treatment timing. The overexposure led to the total breakdown of cell structures, which prevented any useful chromosomal spread with only 8.2±30 (Mean ±SD**)** of average spreads

Table 2 — Effect of colchicine incubation and hypotonic treatment timing on chromosomal spread quality in *Cyprinus carpio* (chromosome count: 2n=100)

| Treatments | Chromosomal Spread Quality |
|---|---|
| T1 | Intact Cell Nuclei prevented the spread of chromosomes |
| T2 | Incomplete cell lysis with unreleased chromosomes |
| T3 | Spreads were unclear |
| T4 | Partial Cell Burst with Indistinct Chromosomal Spread |
| T5 | Adequate chromosomal spreads |
| T6 | Due to high exposure to hypotonic treatment, the chromosomal spreads were unclear and aggregated, correlated with colchicine concentration and incubation time |
| T7 | Optimal chromosomal spreads |
| T8 | Good chromosomal spread with count near to 100 chromosomes visible |
| T9 | Cell destruction due to high hypotonic treatment, correlated with colchicine concentration and incubation time |

Table 3 — Average number of metaphase chromosome spreads among various treatments

| Treatments | Number of metaphasic chromosomal spreads (Mean ±SD) |
|---|---|
| T1 | $0\pm0^{i}$ |
| T2 | $1.79\pm1.48^{h}$ |
| T3 | $24.44\pm4.15^{f}$ |
| T4 | $40.60\pm2.79^{e}$ |
| T5 | $43.13\pm1.99^{d}$ |
| T6 | $45.00\pm2.42^{c}$ |
| T7 | $46.93\pm3.33^{b}$ |
| T8 | $61.4\pm2.75^{a}$ |
| T9 | $8.20\pm3.00^{g}$ |

[Alphabetic subscripts (a-i) indicate significant differences between the treatments ($P$<0.05)]

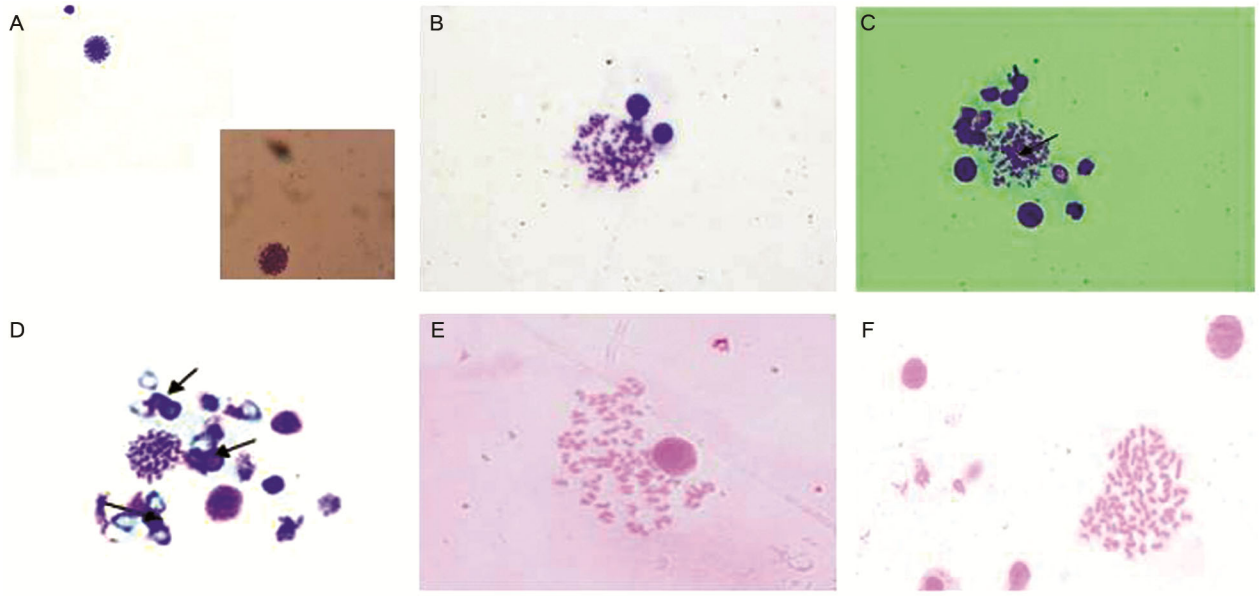


Fig. 1 — Metaphase chromosome spreads of *Cyprinus carpio* (2n=100) at 40×. Images A to F show spreads of chromosomes at various colchicine incubation and hypotonic treatment durations. (A) Intact cell nuclei due to less hypotonic treatment exposure; (B) The cell burst due to moderate colchicine and hypotonic treatment exposure; ( C) Black arrow indicating that due to high exposure to hypotonic treatment, the chromosomal spreads were unclear and aggregated (D) Black arrow indicating the cell rupture; (E & F) Good chromosomal spread

(Fig.1D). Treatment T7 exhibited a distinct chromosomal spread with average of 46.93±3.33 in numbers (Fig.1E). A good chromosomal spreads with complete cell burst were seen in treatment T8 (Fig. 1F), as a significant ($P$<0.05) positive correlation was observed between the colchicines incubation time and the number and quality of metaphasic chromosomal spread at various hypotonic treatment exposure was observed, where the colchicines incubation time and hypotonic treatment duration was for 5 h and 45 min respectively, at constant colchicines concentration of 0.05%, indicating that increase in colchicines incubation time in the treatments, the number of chromosomal spreads also increased (Table 3). Treatment T3 chromosomal distributions were noticeably unclear with 24.44±4.15 (Mean±SD**)** number of chromosomal spreads. The indistinctness of the chromosomal dispersion made it difficult to precisely identify and analyze individual chromosomes due to high exposure to hypotonic treatment along with inadequate fixation or staining conditions. A partial cell burst that left the chromosomal spread unclear happened in T4 (40.6±2.79). Although the chromosomes were partially liberated, they did not disperse uniformly or sufficiently to allow for a useful study. This incomplete bursting suggests that the chromosomal spreads were not as well-defined and distinct as they may have been under the given treatment settings. Table 4 represents that the spreads of the treatments which were treated with one hour of incubation of colchicine (T1, T2 and T3) were statistically different but were least effective in producing spreads with a lowest average value of spreads (8.88±13.62). While, the spreads of treatments which were treated with three hours of incubation of colchicine (T4, T5 and T6) produced the most spreads, as indicated by the mean number of spreads (42.93±2.20), which was the highest of all the groups. The spreads of treatments when treated with five-hour incubation of colchicine (T7, T8 and T9) indicates that the efficacy of spreads was statistically intermediate with a mean number of spreads (38.84±27.50). Table 4 demonstrates that 3 h of colchicine incubation produced the highest number of metaphasic chromosomal spreads followed by 5 h while 1 h was the least effective. Table 5 represents the efficiency of chromosomal spreads under various hypotonic treatment timings with an average number of spreads (29.33±25.5) of the treatments exposed to 35 min of hypotonic treatment (T1, T4 and T7) and were statistically intermediate. While the treatments exposed to 45 min of hypotonic solution (T2, T5 and T8) generated the largest average number of chromosomal spreads (35.44±30.5), suggesting that this duration was the most effective for generating spreads. On the other hand, the treatments with a 60 min hypotonic treatment period (T3, T6 and T9) were the least effective and produced the lowest average number of spreads (25.88±18.4).

Karyotyping is best performed during somatic metaphase, through which one can study the number of chromosomes, morphology and size. Karyotyping has been performed in various ways to date, to visualize fish chromosomes at various developmental stages. The number of cells going through mitotic division is closely correlated with obtaining a desired number of mitotic chromosome spreads. Conversely, species and environmental factors have an impact on mitotic rate[22]. Colchicine as a pretreatment, combined with other kayotyping methods, has revolutionized fish chromosomal research[35,36]. Spindle poison colchicine stops cells in metaphase and is employed in conventional chromosomal preparation[37]. This naturally occurring alkaloid is derived from the *Colchicum autumnale* plant. It binds to tubulin and prevents it from polymerizing, which alters microtubule dynamics and disrupts mitosis. Colchicine induces an irreversible change in tubulin dimers, which results in polymer instability and disintegration[38].Clear chromosomal spread was observed in treatment T8 (Fig. 1F), whereas in treatment T1, the cells did not burst, which resulted in no observable spread (Fig.1A). Treatments T7 and T8 showed clear chromosomal spreads compared to other treatments. Both treatments involved a colchicine incubation time of 5 h, but they differed in hypotonic treatment duration, with 35 min in T7 and 45 min in T8.

Table 4 — Post-Hoc comparison of chromosomal spreads across colchicine incubation timings using Duncan tests. n= 45

| Colchicine incubation timings (h) | Sample size | Average Number of metaphasic chromosomal spreads |
|---|---|---|
| 1 | 45 | 8.88±13.62$^{c}$ |
| 3 | 45 | 42.93±2.20$^{a}$ |
| 5 | 45 | 38.84±27.50$^{b}$ |

[Data expressed as Mean ± SD, Alphabetic subscripts (a-c) indicate significant differences between the treatments ($P$<0.05)]

Table 5 — Post-Hoc comparison of chromosomal spreads across hypotonic treatment timings using Duncan tests. n= 45

| Hypotonic treatment timings (min) | Sample size | Average Number of metaphasic chromosomal spreads |
|---|---|---|
| 35 | 45 | 29.33±25.5$^{b}$ |
| 45 | 45 | 35.44±30.5$^{a}$ |
| 60 | 45 | 25.88±18.4$^{c}$ |

[Data expressed as Mean ± SD, Alphabetic subscripts (a-c) indicate significant differences between the treatments ($P$<0.05)]

This indicates that a 5 h exposure of colchicine concentration can yield high-quality spreads in terms of both clarity and chromosome count, with optimal results depending on the specific duration of hypotonic treatment. In the present study, in treatment T9, the high colchicine incubation timings, resulted in cell rupture, correlated with high hypotonic treatment timing, due to which the chromosomes were condensed and unclear ( Fig. 1D), this study aligns with the findings, as stated by Wood,Cornwell & Jackson[29],that too high concentration of colchicine and prolonged exposure may result in chromosomal condensation. Hence, selecting the right concentration and duration of exposure to colchicines is very important. In the current study, a consistent colchicine concentration of 0.05% was used for the juveniles of common carp across all nine treatments, based on the reference of Jan *et al*.[38] , where the authors optimized the conchicine concentration at 0.05% to prevent the cell division in *Carassius carassius*. Karami *et al*.[12], reported that the optimal colchicine concentration was found at 0.05% among the 0.01%, 0.025% and 0.05%, tested in African catfish (*Clarias gariepinus*) and zebrafish (*Danio rerio*). Similarly, juvenile *Pangasianodon hypophthalmus* and *Clarias gariepinus* showed better chromosome spreads at 0.05% colchicines concentration than at 0.01% and 0.025%, as according to Okomoda *et al.*[39]. Similarly, it is essential to use a suitable hypotonic solution to expand the mitotic cell's nucleus to the point of bursting and spread out the chromosomes after mitotic spindle inhibition[40].Selecting the improper hypotonic solution and incubation time might cause chromosomes to overlap or significantly lose their structure[41]. Hypotonic treatment is necessary to enhance chromosomal spreading. When cell contents are dispersed on slides, hypotonic treatment causes swelling in the cell, which promotes cell disintegration and chromosomal dispersion[38]. The hypotonic solution should be adjusted based on the fish species and/or larval age in order to obtain the desired number of clear chromosomal spreads[12].In the present study, potassium chloride (KCl 0.075M) was used as a hypotonic treatment, based on the study of Okomoda *et al.* [39] , where KCl of 0.075M was more effective than distilled water, where with KCL treatment, there were considerably more distinct metaphase chromosomal spreads in both *Pangasianodon hypophthalmus* and *Clarias gariepinus* than with distilled water. In the current research in treatment T6 and T9 where the exposure timing of hypotonic treatment was for 60 min resulted in unclear chromosomal spreads in T6 (Fig. 1C) whereas in T9 the cell destroyed (Fig.1D), this study aligned with an earlier research, where KCl resulted in significant cell rupture and chromosomal loss in *C. gariepinus* larvae when compared with distilled water. However, when the tissue was incubated for more than one hour in this investigation, chromosomal loss was seen in both hypotonic solutions, but metaphase chromosomes mainly overlapped below this reference point[12]. Despite the similarity of the two studies, the observations differed from the findings of Okomoda *et al.* [39], because in both the studies different developmental stages of the fish was used. In the present study, the hypotonic treatment timing of 45 min was more effective as compared to 35 and 60 min, and resulted in clear chromosomal spread in treatment T5 (Fig. 1B) and T8 (Fig. 1F) as accordance with previous research in which 45 min of hypotonic treatment timing shown to be more effective than 25 and 35 min, also majority of the chromosomes were overlapped at 25 min of hypotonic treatment[38]. According to the results presented in Table 4, the colchicine incubation time of 3 h produced the highest average number of chromosomal spreads of 42.93±2.20 combining treatments T4, T5 and T6 and the hypotonic treatment duration of 45 minutes resulted in the most effective hypotonic treatment with average of 35.44±30.5 spreads combining the treatments T2, T5 and T8 (Table 5). This study aligned with a previous study where a 2 dph larvae of African catfish and 0 dph of zebrafish significantly showed higher number chromosomal spreads at 3 h of colchicine incubation timing[12]. The treatment group T5, in which a 3 h colchicine incubation and a 45 min hypotonic treatment was used, proved to be the most effective in terms of combined effects of the duration of the colchicine incubation and the time of the hypotonic treatment. This combination increased the number of chromosomal spreads. These findings support the idea that chromosomal condensation and spread are influenced by the timing of both colchicine and hypotonic solutions, and that optimizing both of these factors together is essential for a successful metaphase preparation. The right staining solution concentration and incubation time have an impact on the quality of the slides. For 20 min, the chromosomes in the present study were stained with 4–6% Giemsa solution. This is consistent with some of the earlier research that used lower concentrations, like 4–5% of Giemsa solution[42,37].Also majority of the earlier techniques used higher concentrations of Giemsa stain but with lower incubation time[43-45]. Higher concentrations, on the other hand, promoted sedimentation, which may create a dark background and the staining of the chromatid gaps[12]. Physiological factors presumably interfered with the production of well-spread metaphase chromosomes in

many of the samples. More research is necessary to comprehend the function of these components in cytogenetic investigations fully.

## Conclusions

The current study's findings demonstrated that colchicine concentrations of 0.05% number of metaphase chromosomal spread and the quality of chromosomal spreads correlated with KCl treatment for 45 min in *Cyprinus carpio* was found to be more effective than other treatment timings. To obtain high-quality chromosomal spreads, the study concluded by emphasizing the significance of adjusting both colchicine incubation and hypotonic treatment timings. The best results were obtained with treatment T8, which showed ideal and distinct chromosomal spreads. This combination of colchicine incubation timing and hypotonic treatment duration can be considered as a potential standardized method for optimizing chromosomal spreads. However, further research has to be conducted as the spreads in treatment T5 were less when compared with treatment T8 For the purposes of the present study, both T5 and T8 produced satisfactory results and to achieve reliable chromosomal spreads for cytogenetic analysis, it is essential to balance treatment parameters.

## Ethical statement

All applicable international, national, and/or institutional guidelines were followed (Institutional Animal Care and Use Committee of ICAR-DCFR/Ref. No. DCFR/IACUC/25/01/2021/7) for sampling, maintenance, handling and sacrificing of fishes during experiments.

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## Conflict of interest

The authors declare no competing interests.

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