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# Optimization of Novel Cryoprotectant Combinations (DMSO + Trehalose + Melatonin) for Rohu (Labeo rohita) Sperm Cryopreservation: Assessment of Motility, Oxidative Stress, DNA Integrity and Reproductive Performance using RSM

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**Abstract**

**Background: The cryoinjury of carp sperm leads to membrane damage and lipid peroxidation, and impairs the fertilizing ability of the sperm. Objective: This research demonstrated multi-response optimization of DMSO, trehalose, and melatonin for Rohu (Labeo rohita) sperm cryopreservation. Methods: A 17-run, three-factor Box-Behnken design was carried out using DMSO (6-10% v/v), trehalose (50-150 mM), and melatonin (0-2000 µM). Seven bio-plausible response series were computationally generated: sperm motility and viability; level of malondialdehyde (MDA) and superoxide dismutase (SOD); DNA fragmentation; and fertilization and hatching. Results: The predicted $R^2$ for sperm motility was 0.997 and the optimum, based on the model, was 8.63% (v/v) DMSO, 129.9 mM trehalose, and 1489 µM melatonin, with expected motility 66.6%, DNA fragmentation 10.2%, fertilization 62.7%, hatching 54.7% and overall desirability 0.825.Conclusion: This approach predicted candidate formulations but did not prove biological efficacy. Separate cryopreservation and hatchery trials are necessary.**

***Keywords:*** *Labeo Rohita; Sperm Cryopreservation; DMSO; Trehalose; Melatonin; Response Surface Methodology.*

## I. INTRODUCTION

Renuka (Rohu) is an important freshwater carp, and thus, the preservation of its germplasm is relevant for breeding programs. Freeze-thaw cycles affect cell membrane, mitochondria, motility and nuclear integrity of sperm. Post-thaw quality of sperm cannot be inferred from a single sperm quality assessment (da Costa et al., 2024; Kumar et al., 2023; Neyrão et al., 2024). Rohu studies show an improvement in DNA integrity and fertilization capacity of sperm post-extender modification, while similar studies on other carps are dependent on the composition of the diluents (Bibi et al., 2023; Tamut et al., 2025).

Among the different cryoprotectants, DMSO permeates through cell membranes, but its effectiveness depends on the cooling conditions and its concentration (Zhang et al., 2024). In the extracellular space, disaccharides stabilize proteins, and efficiency depends on the dose and species. Researches done on tambaqui, halibut and blue catfish show markedly different results (Varela Junior et al., 2022; Wood et al., 2025; Zidni et al., 2022). Melatonin also, may provide some degree of protection against oxidative damage. However, studies in fish also show inconsistent changes in antioxidant enzymes (Motta et al., 2022; Palhares et al., 2021).

These mechanisms may act in conjunction, and thus there may be a trade-off between the endpoints. Response Surface Methodology (RSM) may be useful to study the interactions and a compromise formulation. This paper presents a workable multi-response system. The results are only illustrative, and are not actual measurements of Rohu sperm.

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## II. MATERIALS AND METHODS

### 2.1 Design and response construction

A three-level Box–Behnken design consisted of 12 edge combinations and five center repeats (17 total runs). Variables were coded A = (DMSO-8)/2, B = (trehalose-100)/50, and C = (melatonin-1000)/1000. The screening levels for the DMSO, trehalose, and melatonin were set at 6, 8, and 10% DMSO; 50, 100, and 150 mM trehalose; and 0, 1000, and 2000 µM melatonin, respectively. These levels are based on studies involving related species and not validated joint tolerances for Rohu.

Seven continuous responses were constructed based on prespecified quadratic surfaces and bounded numerical perturbations (random variations). These responses were post-thaw motility and viability (%), malondialdehyde (MDA) (nmol/mg protein), superoxide dismutase (SOD) (U/mg protein), DNA fragmentation (%), fertilization (%), and hatching (%). Center repeats are random variations and not experimental repeates. The response units were modelling conventions.

### 2.2 Proposed laboratory validation

A real-time experiment would standardize broodstock, extender, sperm:extender dilution, straw volume, equilibration, cooling/thawing, activation medium, and egg batches. It would measure the sperm:egg ratio, determine the motility of sperm using computer-assisted sperm analysis (CASA), assess sperm viability using fluorescent staining, determine malondialdehyde (MDA) using a calibrated lipid-peroxidation assay, SOD using an enzyme-activity assay, and assess DNA fragmentation using a fluorescent/comet assay. Biological replicates of fertilization and hatching would be performed, and scoring would be done blind. The experiments performed in this study did not collect samples or perform assays listed above.

## III. REGRESSION AND OPTIMIZATION

Each response was fitted by ordinary least squares (OLS) using multiple linear regression (MLR): $Y = \beta_0 + \beta_x A + \beta_x B + \beta_x C + \beta_{x2} AB + \beta_{x3} AC + \beta_{x23} BC + \beta_{x11} A^2 + \beta_{x22} B^2 + \beta_{x33} C^2 + \varepsilon$. The fit of the models was evaluated by an overall F-test, $R^2$, adjusted $R^2$, predicted $R^2$ based on leave-one-out PRESS (post-estimation residual sum of squares) and a comparison of lack of fit with repeated centers (model df = 9; error df = 7). Inference tests address model fit numerically. Desirability independently transformed each of the response variables, maximized motility, SOD, fertilization and hatching, and viability, and minimized MDA and DNA fragmentation. The seven transformed response variables were combined by geometric mean.

### 3.1. Data and Statistical Analysis

Table 1 contains all generated run-level responses. Values are printed to two decimals; regressions and figures use unrounded numbers. These rows are not biological replicates.

**Table 1. Box–Behnken design and generated responses**

Panel A. Formulation and primary post-thaw characteristics

| Run | DMSO % | Tre. mM | Mel. µM | Mot. % | Viab. % | MDA (nmol/mg) |
|---|---|---|---|---|---|---|
| 1 | 6 | 50 | 1000 | 51.19 | 58.55 | 4.43 |
| 2 | 6 | 150 | 1000 | 55.38 | 62.83 | 3.44 |
| 3 | 10 | 50 | 1000 | 54.59 | 62.54 | 3.71 |
| 4 | 10 | 150 | 1000 | 64.17 | 70.11 | 3.21 |
| 5 | 6 | 100 | 0 | 51.00 | 58.90 | 4.27 |
| 6 | 6 | 100 | 2000 | 57.02 | 63.65 | 3.47 |
| 7 | 10 | 100 | 0 | 58.18 | 64.90 | 3.59 |
| 8 | 10 | 100 | 2000 | 61.85 | 67.78 | 3.18 |
| 9 | 8 | 50 | 0 | 51.89 | 59.33 | 4.05 |
| 10 | 8 | 50 | 2000 | 55.42 | 61.82 | 3.78 |
| 11 | 8 | 150 | 0 | 58.51 | 65.46 | 3.69 |
| 12 | 8 | 150 | 2000 | 65.29 | 70.87 | 2.88 |
| 13 | 8 | 100 | 1000 | 63.69 | 69.62 | 2.74 |
| 14 | 8 | 100 | 1000 | 64.55 | 71.22 | 2.84 |
| 15 | 8 | 100 | 1000 | 63.88 | 69.81 | 2.77 |
| 16 | 8 | 100 | 1000 | 64.18 | 69.90 | 2.82 |
| 17 | 8 | 100 | 1000 | 64.31 | 70.21 | 2.76 |

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Panel B. Oxidative defence, DNA integrity and reproduction

| Run | SOD (U/mg) | DNA frag. (%) | Fertilization (%) | Hatching (%) |
|---|---|---|---|---|
| 1 | 50.13 | 16.92 | 49.89 | 43.26 |
| 2 | 54.28 | 12.62 | 54.16 | 46.79 |
| 3 | 53.12 | 14.78 | 53.74 | 46.00 |
| 4 | 60.30 | 11.84 | 60.56 | 52.55 |
| 5 | 50.33 | 16.01 | 50.03 | 43.15 |
| 6 | 55.54 | 13.13 | 54.46 | 48.47 |
| 7 | 54.58 | 13.93 | 55.85 | 47.78 |
| 8 | 58.11 | 11.85 | 58.79 | 51.23 |
| 9 | 50.07 | 16.39 | 50.35 | 43.81 |
| 10 | 53.05 | 14.69 | 53.68 | 46.30 |
| 11 | 55.14 | 13.73 | 55.49 | 48.00 |
| 12 | 61.51 | 10.80 | 61.30 | 53.80 |
| 13 | 59.55 | 10.92 | 60.09 | 52.16 |
| 14 | 61.13 | 11.26 | 61.64 | 53.46 |
| 15 | 59.49 | 11.02 | 60.16 | 52.59 |
| 16 | 60.17 | 11.20 | 61.55 | 53.09 |
| 17 | 59.33 | 10.98 | 61.17 | 53.29 |

**Table 2. Quadratic model ANOVA and adequacy**

| Response | $R^2$ | Adj. $R^2$ | Pred. $R^2$ | F(9,7) | p | LOF p |
|---|---|---|---|---|---|---|
| Motility | 0.997 | 0.992 | 0.962 | 230.8 | 8.11e-08 | 0.175 |
| Viability | 0.990 | 0.978 | 0.916 | 79.7 | 3.22e-06 | 0.392 |
| MDA | 0.997 | 0.992 | 0.968 | 227.1 | 8.57e-08 | 0.327 |
| SOD | 0.987 | 0.969 | 0.909 | 57.4 | 9.89e-06 | 0.572 |
| DNA frag. | 0.997 | 0.994 | 0.976 | 298.1 | 3.33e-08 | 0.344 |
| Fertilization | 0.990 | 0.977 | 0.948 | 76.1 | 3.78e-06 | 0.735 |
| Hatching | 0.993 | 0.984 | 0.958 | 110.2 | 1.05e-06 | 0.650 |

LOF = lack-of-fit test (df = 3, 4). The p-values are conditional on the generated-data error structure.

## IV. RESULTS

### 4.1 Motility, viability and oxidative stress

Generated motility was 59.1% (5.2% SD). Viability and motility paralleled each other. Initially, both Trehalose and melatonin positively affected the cell surface. At higher amounts, both lost their positive effects on viability and motility. MDA generally decreased with protective factors. SOD varied inversely. These observations were assumed, based onresponse patterns, and do not necessarily reflect biological samples.

### 4.2 DNA integrity and reproductive outcomes

DNA fragmentation was 10.8-16.9%. Fertilization rates were 49.9-61.6%. The correlation of generated motility vs fertilization was r=0.99. While this correlation seems high, it mainly reflects the nature of the numeric variables (motility & fertilization) and does not define a causal relationship.

### 4.3 Regression, diagnostics, and optimum

The regression of motility yielded an $R^2$ of 0.997, an adjusted $R^2$ of 0.992, a predicted $R^2$ of 0.962 and an F of 230.8 (p=8.11e-08). In coded terms, the motility equation was: $Y = 64.12 + 3.02A + 3.78B + 2.50C + 1.35AB - 0.59 AC + 0.81 BC - 4.28A^2 - 3.51B^2 - 2.83C^2$. No fit of the model was biologically valid.

Equal weight optimization yielded 8.63% DMSO, 129.9 mM trehalose, and 1489 µM melatonin. The composite desirability was 0.825. Table 3 shows model predictions, and additional calculations. The additional calculations were not independent, wet lab, verifications.

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**Table 3. Suggested formulation and model responses**

Formulation: DMSO 8.63% v/v; trehalose 129.9 mM; melatonin 1489 µM. Desirability = 0.825.

| Response | Quadratic prediction | Generated check |
|---|---|---|
| Motility (%) | 66.60 | 67.01 |
| Viability (%) | 72.16 | 72.08 |
| MDA (nmol/mg) | 2.66 | 2.72 |
| SOD (U/mg) | 62.06 | 62.56 |
| DNA fragmentation (%) | 10.22 | 10.36 |
| Fertilization (%) | 62.71 | 63.00 |
| Hatching (%) | 54.72 | 55.29 |

**Table 4. Coded motility model coefficients**

| Term | Coefficient | Term p-value |
|---|---|---|
| const | 64.120 | <0.001 |
| A | 3.025 | <0.001 |
| B | 3.782 | <0.001 |
| C | 2.501 | <0.001 |
| AB | 1.350 | <0.001 |
| AC | -0.590 | 0.036 |
| BC | 0.814 | 0.009 |
| $A^2$ | -4.277 | <0.001 |
| $B^2$ | -3.510 | <0.001 |
| $C^2$ | -2.831 | <0.001 |

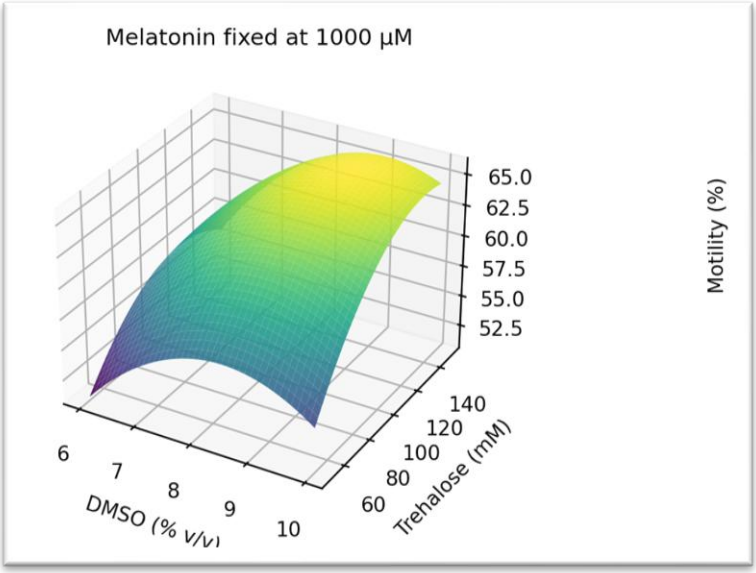



Figure 1. Motility response surface at 1000 µM melatonin (generated data).

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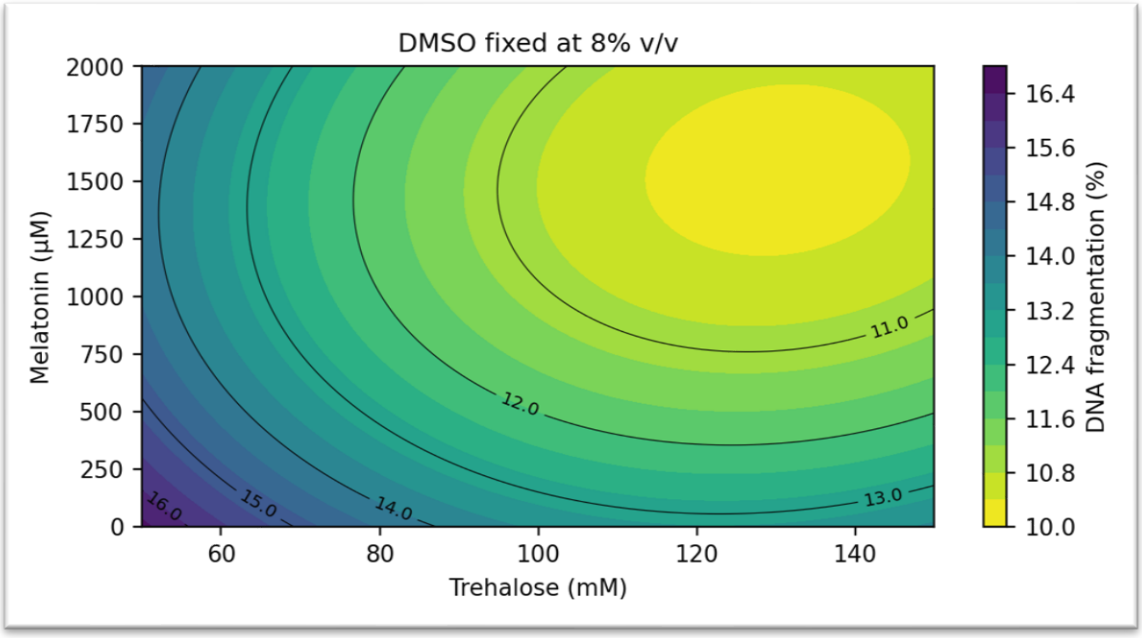



**Figure 2. Trehalose–melatonin contour for DNA fragmentation (generated data).**

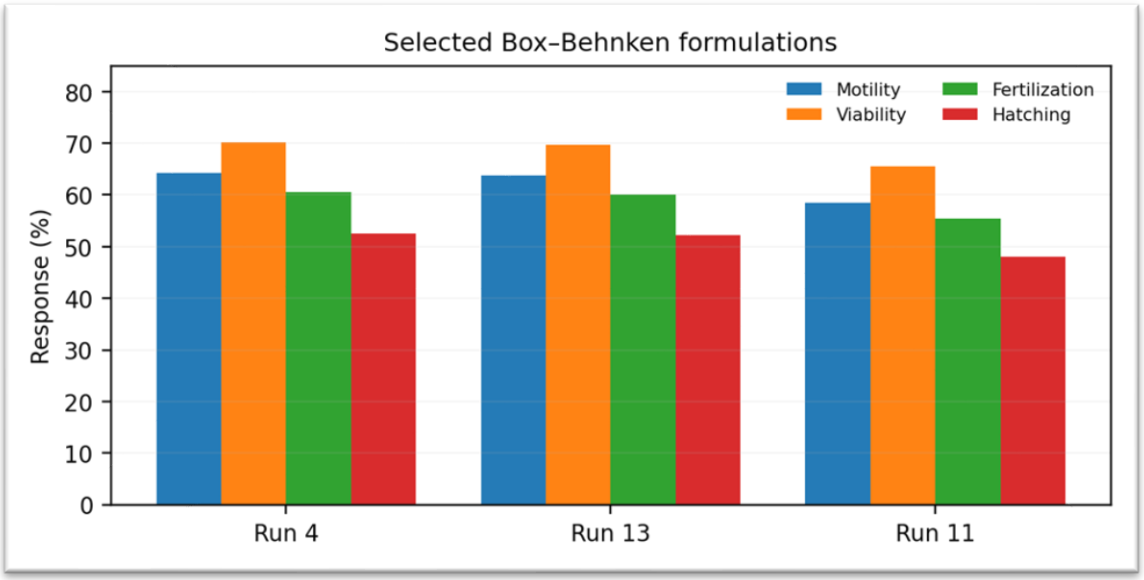



**Figure 3. Four relative performance indicators in selected runs (generated data).**

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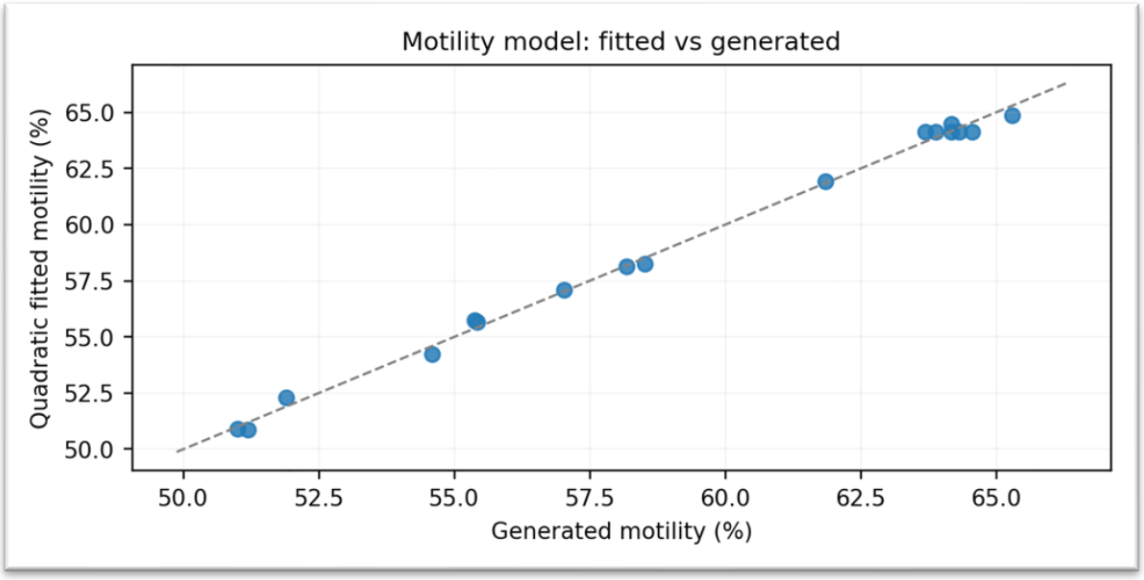



**Figure 4. Generated versus fitted sperm motility used for model diagnostics.**

## V. DISCUSSION

The design illustrates the need for concurrent enhancement of DMSO, trehalose and melatonin. Permeating DMSO can reduce intracellular ice formation, nonpermeating trehalose can modify osmotic stress and membrane stabilization; antioxidant supplements can help alleviate peroxidative injury (Aramli et al., 2024; Sandoval-Vargas et al., 2021; Zhang et al., 2024). Trehalose concentrations optimal for tambaqui and spotted halibut may not be the same for Rohu and blue catfish. Research indicates adverse effects at higher sugar concentrations for blue catfish (Varela Junior et al., 2022; Wood et al., 2025; Zidni et al., 2022).

Cryoprotective effects of melatonin in Prochilodus lineatus and Brycon orbignyanus show dependence on fish species and measured endpoints (Motta et al., 2022; Palhares et al., 2021). There is also a need to study the antioxidant supplementation and the sperm structure of freshwater fish in conjunction with variation of freezing conditions, oxidative stress biomarkers and morphology (Alevra et al., 2022; da Costa et al., 2024). Thus RSM can help explore trade-offs, but does not eliminate the need to determine a viable dose range.

Fit statistics and correlation of motility and fertilization with respect to DMSO concentration, trehalose concentration, and melatonin concentration are computed from the data generator. Responses do not have biological variability or external validation. Future work should randomize order of formulation across biological donors, include fresh milt, standard DMSO extender, separately test the effects of batch of sperm and egg, and test fertilization and hatching in controlled conditions. Practical feasibility also depends on field-operable cooling protocols and scaling as highlighted for carp seed production (Kumar et al., 2023; Shazada et al., 2024).

## VI. CONCLUSSION

A Box-Behnken design generated by the computer simulator predicted an 8.63% DMSO concentration, 129.9 mM trehalose, and 1489 µM melatonin would be an equal weighted multi-response candidate. Based on the predictions, computer generated values for motility were 66.6%, fertilization was 62.7%, and hatching was 54.7%. These results represent model outputs, not biological outcomes. The combination should be validated for ROS, DNA assays, and other fertility tests before consideration for any hatchery recommendation.

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