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Short Communication

Clot contact and temperature: Key factors in serum biochemical changes of rainbow trout (*Oncorhynchus mykiss*)

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Introduction

Evaluating serum biochemical indices is an essential tool in fisheries and biological research for monitoring fish health and physiological performance (Chen and Luo, 2023). Analyzing changes in the concentrations of metabolites, proteins, enzymes, and blood electrolytes can effectively identify clinical or subclinical disorders before visible disease symptoms appear (Chandra, 2024). One of the most critical factors affecting the accuracy and interpretation of these biochemical parameters is the blood sampling and storage conditions, particularly the duration of clot contact with serum and the storage temperature of the samples (Hernandes *et al.,* 2017). Delays in serum separation and improper temperature control can activate intracellular enzymes, alter protein and electrolyte concentrations, and even cause hemolysis of blood cells. These issues can ultimately lead to errors in laboratory results (Simundic *et al.,* 2020). Studies on rainbow trout (*Oncorhynchus mykiss*), a species of high economic value in aquaculture, emphasize the importance of standardizing blood sampling, transportation, and storage methods (Braceland *et al.,* 2017). Due to rainbow trout's sensitivity to physiological and environmental changes, factors such as delays in serum separation and fluctuations in ambient temperature (Majumdar *et al.,* 2019) can significantly impact biochemical test results (Hedayati *et al.,* 2020). Previous reports indicate that prolonged contact of serum with the blood clot and failure to maintain a cold chain during sample transport can lead to misinterpretation of the fish's metabolic or physiological status (Collicutt *et al.,* 2015; Faizo, 2019).

Therefore, understanding the precise effects of clot contact duration and storage temperature on serum biochemical indices in fisheries research is essential (Young *et al.,* 2019). Beyond the significance of research, practical considerations highlight the necessity of adhering to sampling standards. In scenarios where laboratories are far from aquaculture sites, inadequate cooling facilities during transportation, or unforeseen incidents occur, the serum separation process may be delayed, complicating temperature control (Keitel-Gröner *et al.,* 2024). Furthermore, in cases where repeated blood sampling is not feasible or the time interval between samplings is extensive, ensuring proper storage and maintaining the stability of the initial serum sample becomes even more critical. The primary objective of this study is to determine the optimal serum separation time and storage temperature to minimize alterations in the biochemical profile of rainbow trout serum. Similar studies have been conducted on other animal species, such as Turkmen horses (Sadeghian *et al.,* 2021), dogs, alpacas, and sturgeon (Collicutt *et al.,* 2015). The findings of this study are expected to enhance laboratory interpretations, improve the diagnosis and treatment of aquatic animal diseases, and contribute to the standardization of blood sampling and biochemical analysis in fisheries and veterinary sciences. By clarifying the impact of these factors on serum parameter stability, we can minimize potential pre-analytical errors, providing a clear and practical framework for developing standardized guidelines for clinical and research laboratory procedures.

Materials and methods

In this study, 30 healthy rainbow trout (*Oncorhynchus mykiss*) from aquaculture farms in Semnan Province, Iran, were used. Blood samples were collected from the caudal vein with 4-mL syringes and 23-gauge needles, then transferred to seven anticoagulant-free tubes for serum separation. To assess temperature and serum separation time effects on biochemical parameters, samples were stored under refrigeration (4°C) and at room temperature (23±2°C), with serum separated at intervals of 0 (control), 2, 8, and 24 hours. Centrifugation was performed at 3000 rpm for 10 minutes. Serum biochemical parameters, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), glucose, albumin, globulin, and total protein, were measured using commercial kits from Pars Azmun, following the manufacturer's protocol. A JP SELECTA V-1100 spectrophotometer was used for the biochemical analysis.

Statical analysis: The concentrations of the analytes were expressed as means ± standard deviation (SD). To determine the threshold time for the onset of serum biochemical changes at temperatures of 4°C and 25°C following delayed whole blood separation, a repeated-measures analysis of variance (ANOVA) was conducted using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). A multivariate general linear model was utilized to identify statistically significant differences between the two storage temperatures (4°C and 25°C) at each time point (0, 2, 8, and 24 hours). Statistical significance was defined as a p-value of less than 0.05.

Results and discussion

In this study, we examined changes in serum biochemical indices, which included total protein, albumin, globulin, glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP). These were analyzed at two storage temperatures (4°C and 25°C) over four time intervals (0, 2, 8, and 24 hours). The following sections describe the variations in each parameter individually.

Total protein and protein components (Albumin and globulin)

At both storage temperatures (4°C and 25°C), total protein levels showed relatively minor changes during the short intervals (up to 2 hours) and did not present statistically significant differences when compared to the control sample at 0 hours (Fig. 1 A). However, slightly increasing trends were observed over longer durations, particularly at 8 and 24 hours, sometimes approaching or slightly exceeding the initial values. Despite these fluctuations, most variations were statistically insignificant or minor. The findings suggest that prolonged serum contact with the clot may induce slight changes in total protein levels, but these variations were less pronounced than those of other biochemical indices. Recent studies indicate that prolonged serum contact can lead to an apparent increase in total protein due to intracellular protein release, possibly caused by hemolysis or cellular degradation (Magnette *et al.,* 2016). The limited variation in total protein at lower temperatures indicates greater protein stability in cold environments and reduced enzymatic proteolysis (Yang *et al.,* 2023).

The initial decline in total protein levels, followed by a secondary increase, could be attributed to protein consumption or degradation in the early stages, with subsequent re-release in later phases. The more pronounced effect observed at the higher temperature (25°C) further highlights the accelerated impact of proteolytic enzyme activity and potential hemolysis in warmer conditions. Albumin and globulin (Fig. 1 B, C) displayed similar patterns, with minor fluctuations initially but significant reductions or increases over longer durations, depending on temperature. According to Smith and Brown (2020), storing samples at higher temperatures can lead to faster hemolysis and globulin release, whereas at lower temperatures, globulin changes are more gradual and remain within baseline levels over short periods.

Glucose

Initial glucose levels were approximately 148 mg/dL at both temperatures (Fig.1D). At 2 hours, both groups showed a significant decrease to about 93 mg/dL, indicating normal glucose consumption. From 2 to 8 hours, the decline was less pronounced at 4°C (92.93 mg/dL) compared to a sharper drop at 25°C (49.73 mg/dL). After 24 hours, glucose levels at 4°C were around 78.66 mg/dL, while they continued to decrease at 25°C, reaching 23.59 mg/dL.

These results suggest that higher temperatures significantly accelerate glucose consumption and glycolytic processes. Supporting this, Collicutt *et al.* (2015) noted that elevated temperatures enhance glycolytic enzyme activity in red

blood cells, leading to a marked decrease in glucose levels with delayed serum separation. Similarly, Collicutt *et al*. (2015) found that glucose remained stable at room temperature for up to 2 hours but declined sharply between 4 and 8 hours in sturgeon blood samples. To prevent misinterpretation of metabolic status, quick serum separation at low, controlled temperatures is recommended.

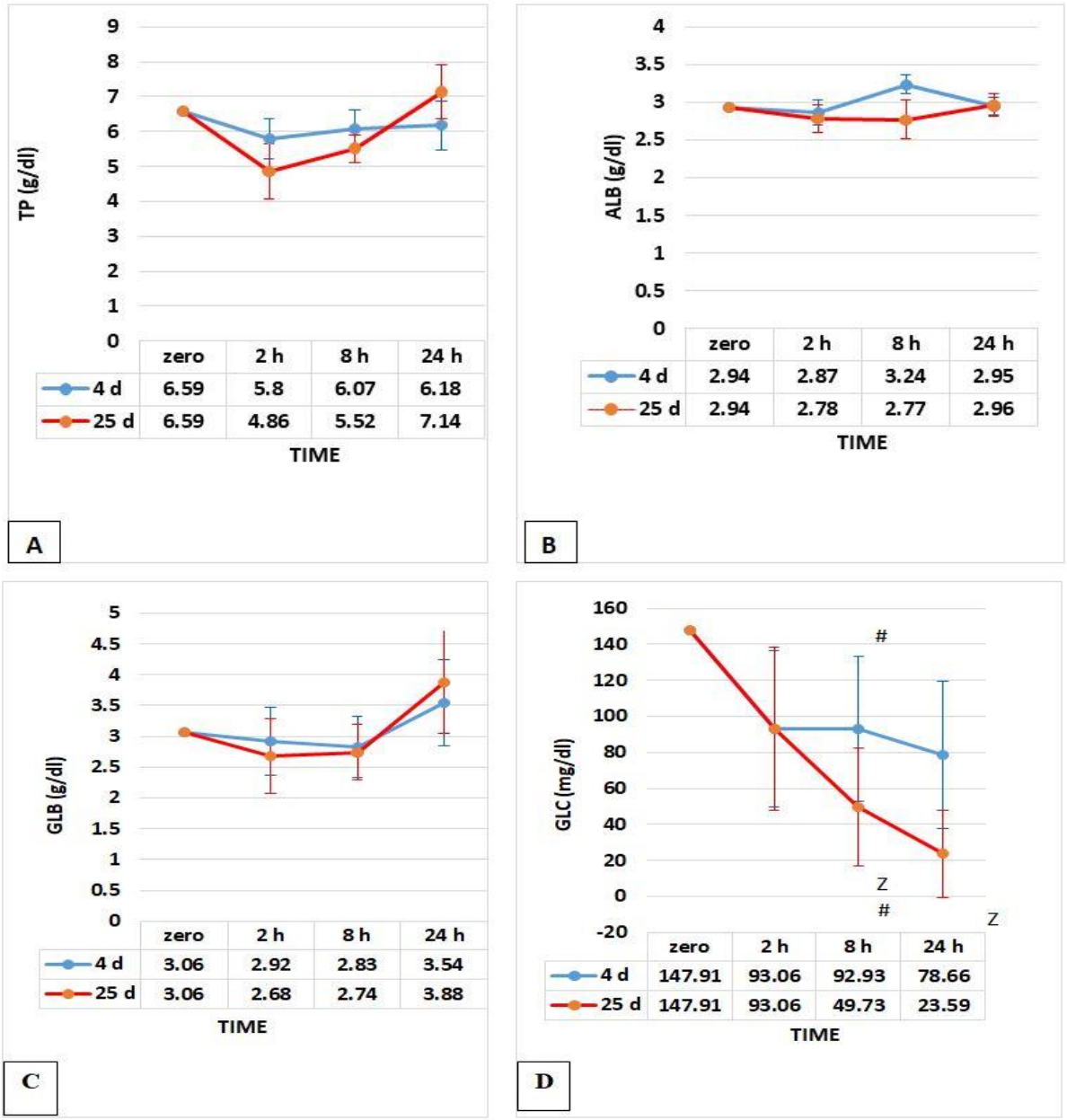

Figure 1: Changes in (A)total Protein (TP), (B) Albumin(ALB),(C) Globulin(GLB) and (D)Glucose(GLC) levels (g/dl) over time in fish samples stored at 4°C(blue line) and 25°C(red line). Error bars represent standard deviations. Z=statistically significant difference between other blood storage times and the control sample (p<0.05), #=statistically significant difference between two consecutive blood storage times (p<0.05).

Liver enzymes

ALT activity

As shown in Figure 2A, ALT activity in fish blood decreased progressively over 24 h at both 4°C and 25°C. At zero time, the

mean ALT was 48.74 IU/L under both conditions. After 2 h, ALT declined to 40.47±3.2 IU/L at 4°C and 41.08±4.1 IU/L at 25°C. By 8 h, the drop was more pronounced (31.58±2.5 IU/L at 4°C; 26.84±3.7 IU/L at 25°C), and at 24 h, ALT measured 29.71±3.0 IU/L at 4°C versus 36.31±2.8 IU/L at 25°C. The decrease from time zero was statistically significant at all subsequent time points (p<0.05), with a larger initial decline observed at 25°C.

AST activity

Figure 2B illustrates AST activity over time. Initial AST was 79.9 IU/L for both temperature groups. At 2 h, AST fell to 47.45 ± 4.5 IU/L at 4 °C and 47.24±5.2 IU/L at 25°C (both p<0.05 vs. zero). Further reduction occurred by 8 h (30.51±2.8 IU/L at 4°C; 20.72±3.1 IU/L at 25°C), with the 25°C group exhibiting a significantly greater decrease than the 4°C group (p<0.05). At 24 h, AST reached 20.19±2.2 IU/L at 4°C and 25.72±3.0 IU/L at 25°C; levels at both 8 h and 24 h remained significantly lower than baseline (p<0.05), and the inter-temperature difference persisted at 8 h (p<0.05).

ALP activity

Changes in ALP activity are presented in Figure 2C. The baseline ALP was 128.14 IU/L in both groups. At 2 h, values were 127.70±6.4 IU/L (4°C) and 131.16±5.9 IU/L (25°C), not significantly different from baseline. At 8 h, ALP peaked at 158.06±7.8 IU/L in the 4°C group (p<0.05 vs. zero) but remained at 105.11±6.2 IU/L at 25°C. By 24 h, ALP declined to 113.72±5.5 IU/L (4°C) and 134.18±6.0 IU/L (25°C), neither differing significantly from baseline. Error bars indicate greater variability for ALP compared to ALT and AST.

In the present study, we evaluated how the interval between blood collection and serum separation ("clot-separation time"), together with subsequent storage temperature (4°C vs. 25°C), influences the stability of ALT, AST, and ALP activities in rainbow trout *(Oncorhynchus mykiss*) blood. Our findings demonstrate that both pre-analytical delay and preservation temperature exert significant, and in some cases biphasic, effects on these hepatic biomarkers.

Delaying serum separation prolongs the contact of enzymes with blood cells and intracellular proteases, accelerating inactivation. Consistent with mammalian models (Jones *et al.,* 2018), ALT and AST activities declined sharply when clot-separation exceeded 2 h, regardless of storage temperature. This early loss likely reflects proteolytic degradation and conformational destabilization while enzymes remain within the cellular milieu. Our data indicate that to minimize artifactual decreases, serum should be harvested within 1 h of collection.

Temperature markedly modulated post-separation enzyme trajectories. At 4°C, ALT and AST exhibited a monotonic decline, reaching ~60% of baseline by 24 h, whereas at 25°C these transaminases showed a pronounced initial drop followed by a partial rebound after 8–24 h.

The late-phase resurgence at room temperature is attributable to gradual hemolysis or thermal damage–induced membrane leakage, releasing residual intracellular enzyme into the serum

(Collicutt *et al.,* 2015). In contrast, cold storage appears to suppress protease activity and stabilize cell membranes, attenuating both degradation and secondary release.

ALP activity responded differently: cold storage produced a transient increase at 8 h; possibly due to de-sequestration from subcellular compartments, before returning toward baseline by 24 h. Under warm conditions, ALP declined initially but then rose above baseline at 24 h, mirroring the hemolytic release pattern observed for transaminases with delayed kinetics. This biphasic ALP response aligns with stage-wise phosphatase liberation described in carp (Warner *et al*., 1978).

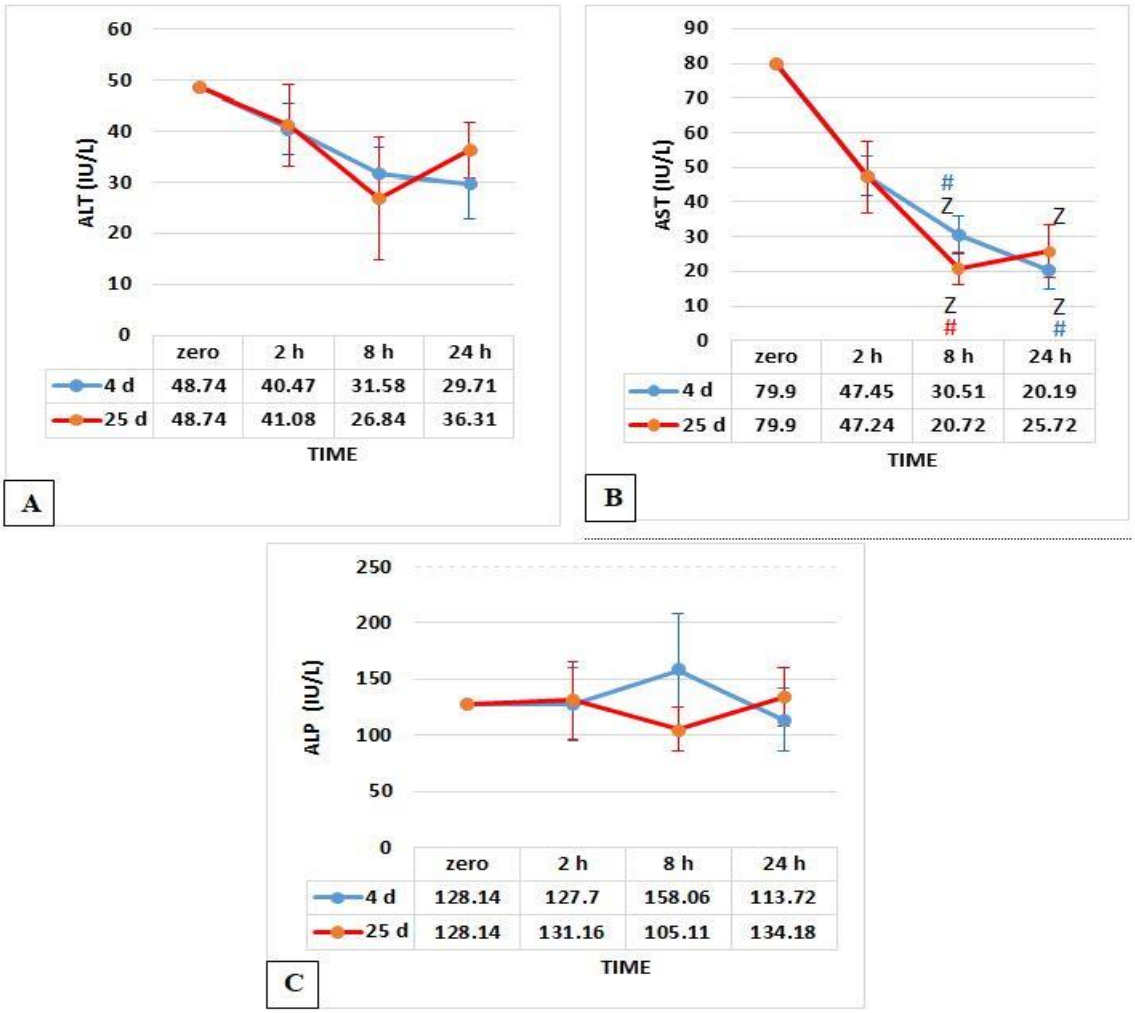

Figure 2: Changes in (A)ALT, (B) AST and (C) ALP levels (g/dl) over time in fish samples stored at 4°C(blue line) and 25°C(red line). Error bars represent standard deviations. Z=statistically significant difference between other blood storage times and the control sample (p<0.05), #=statistically significant difference between two consecutive blood storage times (p<0.05).

Conclusion

Taken together, our results highlight two critical pre-analytical controls for reliable hepatic enzyme assays in rainbow trout:

1-Timely Clot Separation: Serum should be separated within 1 h of collection to preserve true enzyme activity.

2-Cold Storage: Samples must be maintained at 4°C and analyzed within 8 h to avoid both enzyme degradation and artifactual increases from cell lysis.

Neglecting these factors risks underestimating liver injury (due to early enzyme loss) or obtaining spurious elevations (due to hemolysis), potentially leading to erroneous health assessments in aquaculture settings.

Conflicts of Interest

The authors declare no conflict of interest.

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