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REGULAR HUMAN INSULIN SUBJECTED TO AGITATION: IS ITS HYPOGLYCEMIC IMPACT COMPROMISED?

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ABSTRACT

Investigate the effects of intense agitation on insulin stability and its hypoglycemic effect. Samples of regular insulin (Novolin® R100) were subjected to intense agitation for 15 minutes and analyzed using spectrophotometry and in vivo tests on Swiss mice. Turbidity measurements were conducted at various time points, and the agitated insulin was administered to mice to assess its effects on blood glucose levels. Despite the agitation, insulin retained its hypoglycemic effect in the in vivo experiments. Spectrophotometric assays showed a significant increase in absorbance over time (a 6.7-fold increase with the addition of bromophenol blue dye, $p<0.05$). However, the reduction in blood glucose levels in Swiss mice treated with agitated insulin was not significantly different from those treated with non-agitated insulin ($p>0.05$). These findings suggest that, despite potential conformational changes under agitation, insulin retains its hypoglycemic efficacy. This highlights the robustness of insulin's stability. Further studies are recommended to investigate the long-term stability of insulin formulations to ensure optimal management of diabetes mellitus for both healthcare professionals and patients.

Keywords: human insulin; agitation; protein stability; hypoglycemic efficacy; diabetes mellitus

1. INTRODUCTION

Diabetes mellitus (DM) is currently considered a global epidemic, creating a key challenge for health systems around the world as preventive strategies for improving conventional treatments (Fu, Gilbert and Liu, 2013; Banday, Sameer and Nissar, 2020; Arroyave, Montaño and Lizcano, 2020; Vargas et al., 2023; Genuth, Palmer and Nathan, 2024). This chronic metabolic disorder is characterized by insufficient insulin production or reduced sensitivity to insulin in target tissues (Solis-Herrera et al., 2000; Matijević et al., 2023). In this context, insulin replacement therapy is a cornerstone in managing diabetes, and the stability of insulin formulations is crucial to ensure their therapeutic efficacy (Hegele and Maltman, 2020; Ahmad et al., 2023).

Agitation is common during the handling and administration of insulin (Malik and Roy, 2011). Although several types of insulin are available with different durations of action, the impact of agitation and its physiological effectiveness are not yet clear (Sluzky et al., 1991;

Malik and Roy, 2011; Das, Shah and Saraogi, 2022). In this context, to better understand the stability of insulin, the main hormone replacement, this disease becomes relevant.

This study aimed to investigate the impact of agitation-induced protein denaturation on insulin stability and its hypoglycemic efficacy in a murine experimental model, providing valuable insights into optimizing insulin formulations to ensure their hypoglycemic effect in diabetic patients.

2. METHODS

Insulin agitation

Samples of regular insulin (Novolin® R100) were intensely agitated using a manual shaker for 15 minutes. The preparation of agitated insulin followed a modified procedure based on the methodology proposed by Malik and Roy (2011). The insulin solution was prepared with freshly prepared sodium phosphate buffer (0.05 mol/L, pH 7.4) at a concentration of 0.5 mg/mL, determined using an extinction coefficient of 1 mg/mL at 276 nm, as described by Malik and Roy (2011). A volume of 1 mL of the insulin solution was aliquoted into 10 mL glass vials, after which the samples were set aside for spectrophotometric and physiological analyses.

Spectrophotometric assays

The samples were analyzed in triplicate for turbidity using turbidimetry at various time intervals (0, 15, 30, 45, 60, and 90 minutes). The turbidity of the agitated suspensions was measured by determining the absorbance at 600 nm, both with and without the addition of 100 µL of 1% bromophenol blue dye. Control samples, incubated without agitation, were evaluated at the same time points for comparison.

Animals

Experiments were conducted in male Swiss mice (30g), maintained in the animal care facility at the Experimental Monitoring Laboratory of Vila Velha University (UVV), Espirito Santo, Brazil. The mice were provided a normal chow diet and water ad libitum, and housed under standard conditions with a controlled temperature (~22ºC), humidity (~60%), and a 12/12-h light-dark cycle. All biological assays were realized in accordance with the guidelines for the care and handling of laboratory animals as recommended by the National Institutes of Health (NIH) and the Brazilian Society of Experimental Biology. All protocols were previously approved by the Ethics, Bioethics, and Animal Welfare Committee of Vila Velha University (EBAW-UVV # 403/2016).

In vivo experiments

The protocol for the insulin tolerance test was adapted from previous studies (Brüning et al., 1997; Ayala et al., 2010). The agitated insulin solution, at different time intervals, was administered to mice that had been fasted for 5-6 hours to assess its effects on blood glucose levels. An aliquot of insulin was withdrawn from the stock vial using a 27 G needle attached to a syringe and transferred into a sterile tube. Then, 25 μL of the stock solution was diluted in 10 mL of sterile saline (0.9% NaCl), and sterilized by filtration, to achieve a concentration of 2.5 U/10 mL. The mice were immobilized, and a small cut was made at the tip of the tail to allow for blood glucose measurements using a glucometer. A dose of 100 U/kg of regular insulin was administered via intraperitoneal injection, with blood glucose levels measured at 0, 10, 30, 60, and 90 minutes. After the experiment, 0.5 mL of 10% glucose solution was injected intraperitoneally to reverse hypoglycemia in the animals.

Statistical analysis

The results were expressed as mean ± SEM (standard error of the mean). The means of the values comparing different time intervals were statistically analyzed using one-way or two-way analysis of variance (ANOVA) for completely randomized designs, followed by post hoc Tukey's or Bonferroni's test for repeated measures, using PRISMA software (version 5). Differences were considered significant when $p < 0.05$.

3. RESULTS

Through turbidimetric evaluations using spectrophotometry, a significant increase in solution turbidity was observed when agitation was applied at successive intervals (0, 15, 30, 45, 60, and 90 minutes). Additionally, the inclusion of bromophenol blue enhanced the analytical sensitivity of the assay, particularly after 30 minutes, resulting in a 6.7-fold increase in the area under the curve ($p < 0.05$), as shown in Figure 1.

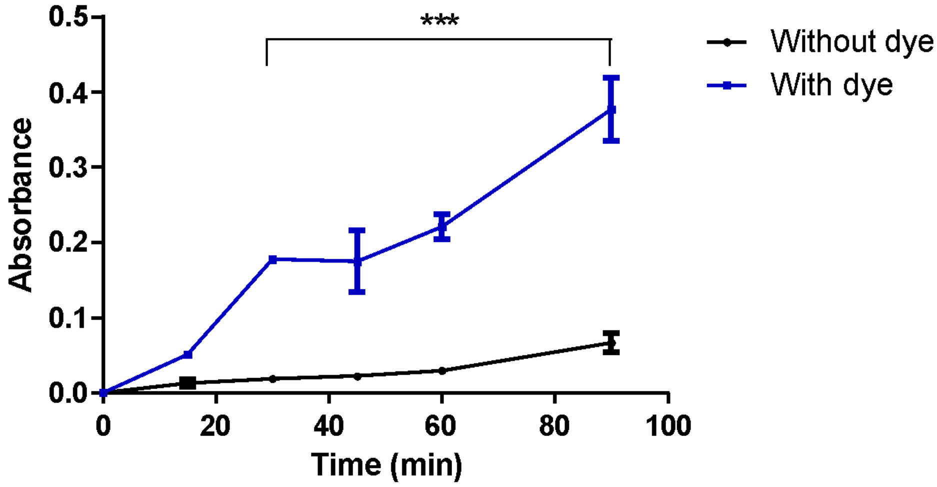

Figure 1: Evaluation of insulin turbidity without bromophenol addition (black line) and with bromophenol addition (blue line) at successive intervals. *** p < 0.01 (Two-way ANOVA, Bonferroni post-hoc)

Despite the increasing variation observed through turbidimetry, our in vivo results in mice showed that insulin agitated for 90 minutes did not exhibit compromised biological function ($p > 0.05$), as shown in Figure 2. This was demonstrated through graphs presenting both absolute and relative glycemia values.

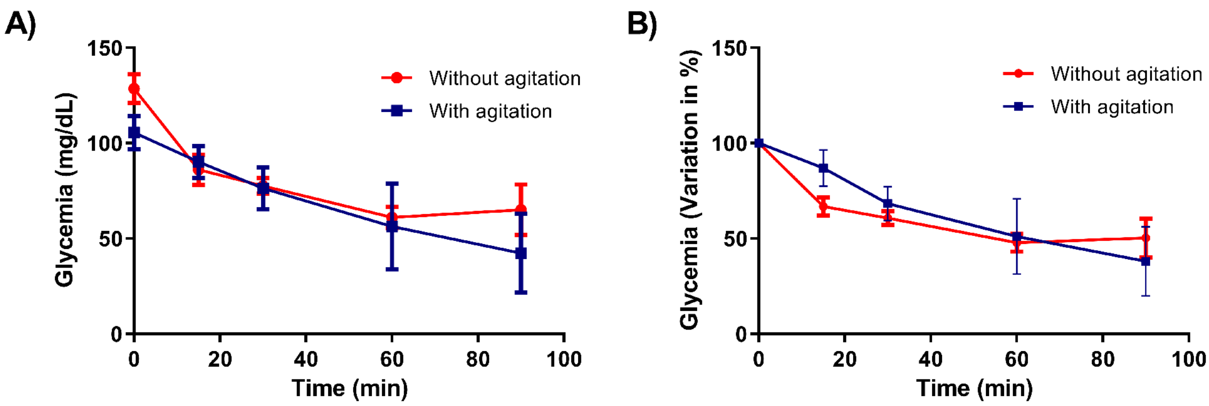

Figure 2: Evaluation of insulin tolerance test by analyzing the hypoglycemic efficacy of non-agitated insulin (red line) and agitated insulin (blue line). Data are presented in absolute (A) and relative (B) values.

4. DISCUSSION

In this study, turbidimetric evaluations revealed a significant increase in insulin turbidity due to agitation and the presence of bromophenol blue, enhancing analytical sensitivity by 6.7 times. Notably, insulin agitated for 90 minutes preserved its biological function, with no compromise in glycemic levels. Earlier studies have shown that the formation of irreversible aggregates of protein could lead to issues such as immunogenicity (Fineberg et al., 2007; Sauerborn et al., 2010) and low bioavailability (Pezron, Mitra and Mitra, 2002). Based on these biochemical theories, we also hypothesized that agitation of insulin during storage, transportation, and handling by users also could contribute to its denaturation. Indeed, current literature and even national healthcare guidelines describe the loss of efficacy due to agitation without quantified references (Malik and Roy, 2011; Das, Shah and Saraogi, 2022), justifying the relevance of this study.

We aimed to comprehend the nature of insulin under agitation. Reliable techniques were employed to indirectly confirm the presence of aggregates in the solution and investigate how this perturbation could alter its physiological characteristics (Sluzky et al., 1991; Malik and Roy, 2011). Moreover, it is known that the agitation of any protein can induce molecular realignment at the air-water interface, exposing the protein's hydrophobic inner core to the air, which may serve as a nucleation site for aggregation through hydrophobic interactions (Malik and Roy, 2011; Das, Shah and Saraogi, 2022). However, the impact of physical changes accompanying the denaturation process resulting in the formation of aggregates in insulin are still poorly understood (Das, Shah and Saraogi, 2022). Our innovative results showed that agitated insulin could be readily identified by an accessible and reliable method, using turbidimetry. Additionally, the addition of bromophenol blue significantly increased the analytical sensitivity of the test. Interestingly, we demonstrated, for the first time, that even under exhaustive agitation, insulin retains its hypoglycemic efficacy.

According to previous studies, protein aggregation can arise through various distinct mechanisms (Malik and Roy, 2011; Das, Shah and Saraogi, 2022). The, a thorough understanding of the aggregation mechanism can aid in formulating effective methods to prevent or eliminate aggregates. Insulin serves as an example of a therapeutic protein that readily associates to form reversible oligomers, which over time can become irreversible larger aggregates (Malik and Roy, 2011). Moreover, other mechanisms, such as those induced by heat or shear stress, can trigger initial conformational changes. On the other hand, it is known that aggregation can be inhibited by excipients or conditions stabilizing the initial conformation, thus preserving insulin's biological activity. Certain "protein stabilizers," such as sucrose, polyols, specific amino acids, and salts, promote enhanced structural stability against various environmental stresses that cause unfolding. In addition, the thermodynamically unfavorable interactions of these substances with protein surfaces favor minimal surface area and, consequently, the native structure, reducing aggregation (Sluzky et al., 1991; Malik and Roy, 2011). In our study, it was diluted in a zinc-rich solution, which may also have contributed to preventing protein aggregation.

The measurement of solution turbidity showed a significant increase with agitation at progressively longer time intervals (until 90 minutes). The present study demonstrated that this method provided a useful substitute for visually recognizing uniform opalescent suspensions, as it proved more advantageous, less time-consuming, and potentially more reproducible, precise, and objective. Additionally, the addition of bromophenol blue enhanced the analytical sensitivity, increasing absorbance approximately fivefold compared to insulin readings without the dye, while maintaining solubility in the neutral pH insulin solution (Kreft and Kreft, 2007).

The insulin tolerance test was evaluated using samples with the longest agitation time (90 minutes). Since no efficacy changes were observed during this period, observations of other time points became clearly unnecessary. Therefore, we found that aggregate formation did not influence the insulin effect. Consequently, no loss of hypoglycemic efficacy was observed after agitation.

Due to logistical reasons with experimental animals, this procedure was performed with a 24-36 h interval between sessions. Therefore, we suspected that the same resting sample could recover its native form, justifying the preservation of the hypoglycemic effect. By conducting successive turbidimetric measurements of the same samples at 2, 4, and 24-hour intervals, we concluded that insulin could not undergo renaturation and return to its original solubility, evidencing an irreversible reaction. However, through our study, we found that even with the formation of irreversible aggregates, insulin's efficacy remained intact, as the hypoglycemic level decreased normally. Despite this seemingly paradoxical functional preservation, little is known about how insulin binds to its receptor to trigger its effect. Its active conformation and contact points with its receptor are still unclear (Hua, 2010)). We can infer that mechanical agitation of insulin did not compromise its biological effect, as it did not disrupt the insulin-receptor binding points. Additionally, the argument that the three-dimensional structure is highly stable, supported by its small size and the presence of disulfide bonds, may ensure its functionality even under agitation.

CONCLUSION

We concluded that agitated insulin can be effectively detected using turbidimetry, which measures turbidity at various intervals. The addition of bromophenol blue significantly enhanced the analytical sensitivity of the test. Paradoxically, in vivo assays showed that agitated insulin maintained its biological function despite the formation of persistent precipitates, which did not influence its efficacy. These findings offer valuable insights for future research on insulin stability and optimizing insulin therapy for healthcare professionals and patients.

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