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Assessing Hypoxia Tolerance using Rapid Colorimetric Testing of Hypoxia Inducible Factor-1 (HIF-1)

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ABSTRACT

The body reacts by producing a protein called hypoxia-inducible factor (HIF), which triggers a series of physiological changes and serves a central role in the hypoxia response. Its activity is regulated by the oxygen-dependent degradation of the HIF-1α protein (HIF-1A gene). Therefore, the effects of low oxygen tension in hypoxia were explored using rapid and colorimetric tests. The development of the sensor provided more sensitivity for detecting hypoxia by generating a calibration of optimized parameters such as reagent concentrations, reagent volumes, and device dimensions. There is a high sensitivity and specificity in detecting the changes of HIF-1α protein hypoxia using the feasibility hypoxia test. This would enable point-of-care (POC) testing and individual self-administration, resulting in faster and more accurate results that can be used for a robust diagnostic approach and enhanced health surveillance.

1. Introduction

Aircrews exposed to high altitude with longer durations are at a higher potential risk for detrimental health consequences. The altitude and other environmental features are of concern for aircraft safety [1]. For safety reasons, proper acclimatization is important for those traveling to high altitudes. While the effect is most dramatic at altitudes greater than 8000 feet (2438 meters) above sea level, it becomes noticeable even at 5000 feet (1524 meters) above sea level. Among other important changes (e.g., decreases in temperature and ambient humidity), the defining environmental feature at high altitude is a drop in barometric pressure, which causes a decrease in the partial pressure of oxygen at every point along the oxygen transport cascade from ambient air to cellular mitochondria. Subsequently, there is also a decrease in PO2 at every point along the oxygen transport cascade from inspired air to the alveolar space, arterial blood, tissues, and venous blood. The higher the elevation attained and the longer the duration of spaceflight, the greater the drop in PO2 in the human body [2].

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Ascent to high altitude is associated with physiological responses that counter the stress of hypobaric hypoxia by increasing oxygen delivery and altering tissue oxygen utilization via metabolic modulation. At the cellular level, the transcriptional response to hypoxia is mediated by the hypoxia-inducible factor (HIF) pathway and results in the promotion of glycolytic capacity and the suppression of oxidative metabolism [3]. Hypoxia inducible factor-1 (HIF-1) plays a key role in oxygen homeostasis by facilitating oxygen supply to the tissues under hypoxic conditions, as during acclimatization to hypobaric hypoxia or inflammation molecular response. HIF-1 is found in almost all body tissues. Under normoxic conditions, it is degraded through hydroxylation, but it does not undergo degradation in the presence of hypoxia [4].

Under normal oxygen conditions, a protein called von Hippel-Lindau (VHL) undergoes chemical modification, enabling it to bind to hypoxia-inducible factor (HIF), thereby marking HIF for degradation. However, when oxygen levels are low, VHL is not modified and therefore cannot attach to HIF; as a result, HIF persists. Monitoring HIF levels within cells and tissues provides a measure of the extent of hypoxia and hypoxic gradients. Hence, detection of hypoxia often relies on strategies for the detection of HIF protein expression. There are several techniques to detect hypoxia by targeting HIF protein, including western blot, immunoassay (ELISA, Luminex), immunochemistry (IHC), and flow cytometry. These techniques require specialized equipment and complex data analysis [8]. It also takes time to get the results. Due to the importance of early detection of hypoxia to avoid acute symptoms and the limitation of detection by using the current techniques, we developed lateral-flow assays (LFAs) to early detect hypoxia via targeting HIF protein [9].

LFAs are quick, simple, and cheap assays to analyze various samples at the point of care or in the field, making them one of the most widespread biosensors currently available [10]. Lateral-flow assays (LFAs) have become one of the most widely used point-of-care (PoC) sensors in a variety of disciplines ranging from diagnostics to environmental and safety analysis due to their ease of use and low cost [11,12]. Their success lies in their general design, which has remained almost unchanged since their first use as pregnancy tests in the 1970s. Although all LFAs simply rely on capillary forces to move the sample along a test strip to generate a measurable signal, their fabrication is far from straightforward [13,14]. From a user perspective, the operation of a well-designed LFA is strikingly simple: the assay is executed by adding the sample onto the lateral-flow strip (LF strip), and after a short incubation time, the positive or negative outcome of the test is revealed by the appearance of a line. In this study, we established lateral-flow assays (LFAs) to detect hypoxia via targeting the HIF protein.

2. Materials And Methods

a. Reagents

AuNP solution, HIF-1a HRP, BSA, DAB detection kit, Anti-HIF-1 alpha antibody [mgc3], Recombinant Human HIF-1 alpha protein, Goat Anti-Mouse IgG H&L, Nitrocellulose Transfer Membrane- 0.45 µm, Tween-20, Filter paper chromatography, Sucrose-AR Sodium dodecyl sulphate, methanol, Glycine, Tris base, phosphate buffer saline, sodium chloride, boric acid, sodium tetraborate, isopropanol, glass fiber, Whatman filter paper.

b. Dot blot enzyme immunoassay

The assay was performed using Nitrocellulose Transfer Membrane- 0.45 µm. The membrane was then cut into the appropriate size. The membrane was then put into a plastic tray and treated with a

blotting buffer. Then the membrane was put on layers of wet filter paper. Using a narrow-mouth pipette, slowly spot the sample onto the nitrocellulose membrane at the center of the grid. Non-specific sites were blocked after the membrane had dried by shaking in a blocking solution that included BSA prepared in PBS-Tween20 in a tray for 1 h at room temperature. The membrane was later transferred to dry tissue paper and dried at 37 °C for half an hour. To each spot of test sample, anti-HIF-1a Monoclonal antibody conjugated was added to HRP at a predetermined dilution in BSA. The membrane was left at room temperature for two hours under moist conditions and washed with PBS Tween 20 (PBST). Freshly prepared diamino benzidine was added, and the membrane was kept in the dark for ten minutes. The reaction was terminated using tap water. A distinct dark-brown colored spot was observed with positive control.

c. Component of lateral flow immunoassay (LFIA) strip

The LFIA is composed of four parts, including a sample pad, a conjugated pad, NC membranes, and an absorbent pad. The sample pad, which contains a buffer system and some surfactants, is specially designed for oral fluid collection and quickly transports oral fluid into the conjugate pad. The antibody–AuNP conjugates were sprayed on the conjugate pad to react with the sample and be released from the pad and enter the NC membrane coated with the Antibody HIF1a protein on the Test line and goat anti-rabbit IgG antibodies on the control line. The absorbent pad is a filter paper located at the end of the strip; it maintains the capillary flow and discards the unbound detector particles away from the test line and control lines, thereby lowering the background and enhancing assay sensitivity.

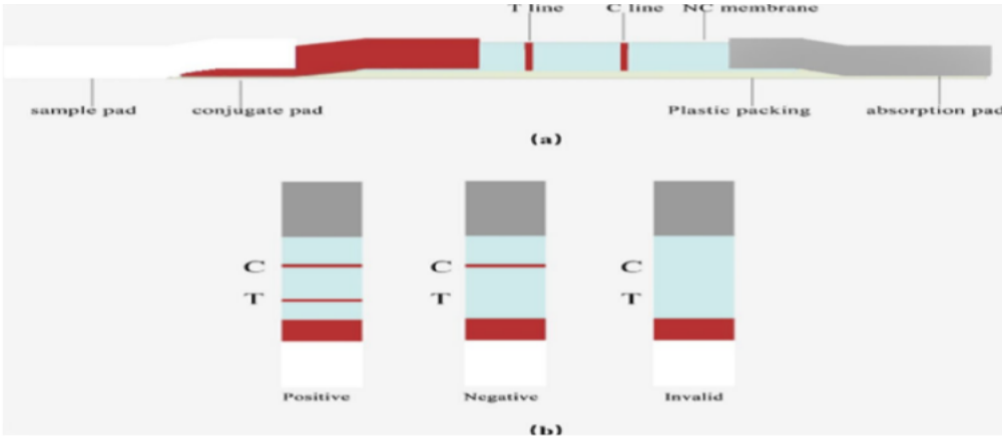

Fig. 1. Images and design of the lateral flow strip

(a) The lateral flow strip in vertical position. The test assembly consists of a sample pad, a conjugation pad, a nitrocellulose membrane, and an absorbent pad. (b) The lateral flow strip inside view

d. Optimizing AuNPs for detection

To test the aggregation of nanoparticle-bioreceptor, AuNPs solution was set at pH 8.5 using borate buffer. Five dilutions of anti HIF-1a (200, 150, 100, 50, and 0 µg/ml) were prepared in Milli-Q water. In a 96-microwell plate, each anti-HIF-1a dilution was added into AuNP and incubated for 20min at 600 rpm at room temperature. The aggregation rate can be qualitatively assessed by the naked eye in the presence of high salt concentration (10%NaCl).

e. Preparation of gold nanoparticles-antibody conjugates

A predetermined concentration Antibody HIF 1A (which was optimized in the previous step) was added to 20 nm AuNPs solution and then incubated for 20 min at room temperature by using a thermoshaker at 650 rpm. BSA was added to the solution for 20 min at room temperature at 650 rpm. The solution was centrifuged at 14000 rpm for 20 min at 4 c, and then the supernatant was discarded. The pellet was then resuspended in conjugate pad buffer.

f. Preparation of LFIA strip

Firstly, conjugate pad buffer with gold nanoparticles-antibody conjugates that prepared in the previous step, was stripped on the conjugate pad until fully colored and then was dried in the oven at room temperature for 2 hrs. To prepare the coated nitrocellulose membrane, Antibody HIF 1A was prepared in the stripping buffer and sprayed in the test line. While Antibody IgG was also added to the stripping buffer and then sprayed in the control line. To fix the antibodies, the membrane was incubated at room temperature for 2 hours in the oven. Both pads were soaked into sample and absorbent pads buffer until fully wet and they were dried in the oven at 37 C for 2 hrs.

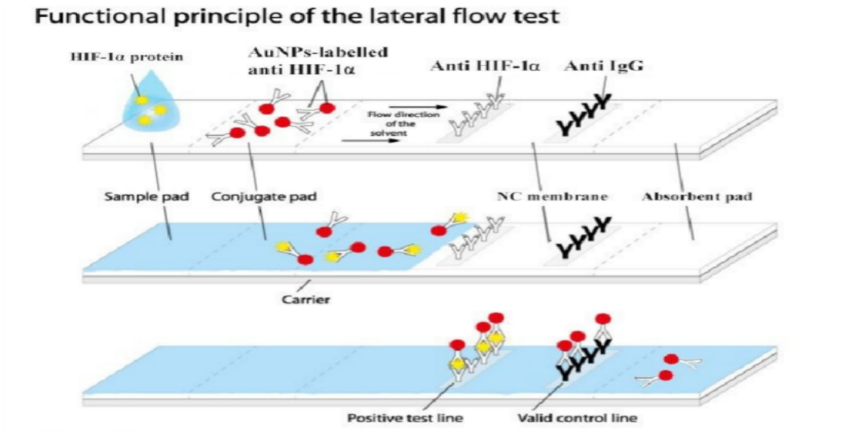

Fig. 2. The mechanism of LFIA strip

(a) shows HIF1A protein on the sample pad, gold nanoparticles-anti HIF 1A conjugate on the conjugate pad, and Anti HIF1A and anti IgG immobilized at T and C line on membrane respectively. (b) the sample fluid simply flows through the sample pad and directly enters the conjugate pad where AuNPs-anti HIF1A. HIF1A protein binds to the AuNPs-anti HIF1A and goes toward the nitrocellulose membrane. (c) AuNPs-anti HIF1A conjugated HIF1a protein only binds to T line. The free AUNPs-anti HIF1a binds to anti IgG. The unbounded AuNPs-anti HIF1A discards via absorbent pad.

3. Results And Discussion

1. Dot plot

The dot blot immunoassay has been used as a common protein detection method for detection of disease markers. Evaluation of the detection of HIF-1a was done by dot blot tests. Here, A nitrocellulose membrane was functionalized with a different concentration of the target molecules in the form of dots immobilized in different locations along the membrane. The remaining non-specific sites were blocked and then the membrane was exposed to a detection solution. Preliminary

results were obtained by using the well-known Horseradish peroxidase (HRP)-coupling anti-HIF1a antibody. HRP reacts with diaminobenzidine (DAB) to produce a visible, brown-colored product. On the basis of the immunoassay with HRP as labels, we conclude that the immobilized proteins were able to capture antibody-conjugated particles because of the antigen-antibody interaction. Thus, the more proteins spotted on the membrane, the greater the number of particles that will accumulate on the spot, and the colorimetric intensity of the spots is thus dependent on the concentration of proteins. However, the area reduction is due to the high affinity of the protein to the nitrocellulose membrane, avoiding the protein spreading during the solution deposition (Figure 3).

Fig. 3. Dot-blot immunoassay on NC membrane for rapid detection of HIF-1a protein a) shows the higher protein amount, and b) shows the is the lower protein amount

2. The gold aggregation results

As shown in Figure 4, no color was observed at 0 concentration of anti-HIF-1a while increasing concentration to 50 µg/ml of anti-HIF-1a, shows a light pink color due to the aggregation of gold nanoparticles with the antibody in the presence of NACL solution. The gradient color was also observed by the naked eye when increasing anti-HIF-1a concentration from 50 µg/ml to 200 µg/ml. This experiment was conducted to determine the low concentration of anti-HIF-1a that would be used for treatment of the conjugate pad in the lateral flow strip preparation.

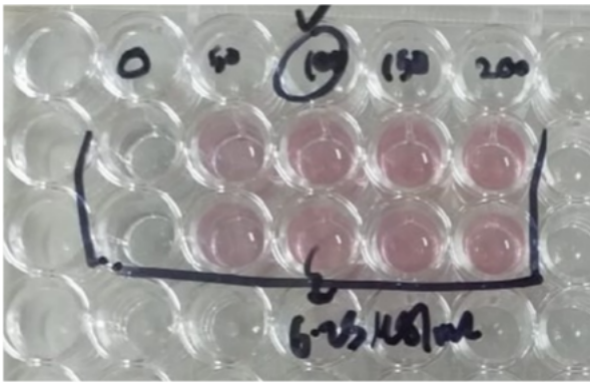

Fig. 4. The image shows the gold nanoparticles 20 nm in diameter, aggregation with anti-HIF-1a protein

The gradient color showed that the different concentrations of anti-HIF1a protein were used. The experiment was done to estimate the minimum antibody concentration used for the lateral flow strip.

3. Lateral flow strip results

The objective of this project is to design a lateral flow strip for the detection of HIF-1a protein. As shown in Figure 5a, the final design of the assembled strip for detection of HIF-1a protein after treatment of both conjugate and sample pads, immobilized anti-HIF-1a protein in T line and anti-IgG in C line, and sprayed the glass fiber with gold-labeled anti-HIF-1a protein. Figure 5b shows the fully strip with positive results for detection of HIF-1a protein with the presence of the red color in both T and C. Figure 5C shows no band was seen in T line and red band is visible in the C line which confirmed the negative results when using only PBS as a control instead of using HIF-1a protein. The results confirmed that we succeeded in optimizing and designing the lateral flow strip for detection of HIF-1a protein.

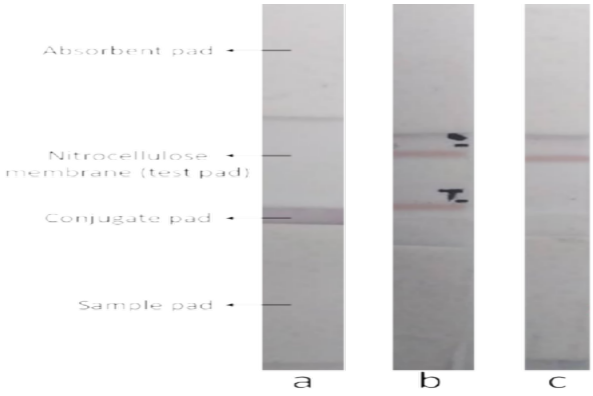

Fig. 5. Image shows lateral flow strip test

(a) shows the design of the lateral flow strip after treatment. In (B), a sample that demonstrates a positive result for the HIF1a protein r with the red color in both test and control lines. (c) shows a negative result when there is no band in the T line and a clear band in the C line.

4. Conclusion

This test would enable point-of-care (POC) testing and individual self-administration, resulting in faster and more accurate results that can be used for a robust diagnostic approach and enhanced health surveillance to detect hypoxia.

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