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Evaluation of Rapid Colorimetric Lateral Flow Sensor for Hypoxia-Inducible Factor-1 Alpha Detection in High Altitude

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ABSTRACT

Hypoxia is a critical physiological risk in high-altitude aviation and aerospace environments, where early detection is essential to prevent cognitive impairment and loss of consciousness among aircrew. Hypoxia-inducible factor-1 alpha (HIF-1α) is a molecular biomarker that is rapidly upregulated under low-oxygen conditions. This study aimed to develop and evaluate a rapid, non-invasive lateral flow immunoassay (LFIA) for colorimetric detection of salivary HIF-1α. A total of 24 saliva samples were collected from participants at the Institute of Aviation Medicine and divided into two groups: sea level (control) and simulated altitude exposure at 10,000 feet. Salivary HIF-1α was detected using the developed LFIA sensor, while peripheral oxygen saturation (SpO_2) was concurrently measured using fingertip pulse oximetry to confirm hypoxic status. All participants tested negative for HIF-1α at sea level, whereas positive LFIA results were observed in all participants after altitude exposure, indicating 100% sensitivity under the study conditions. Mean SpO_2 decreased significantly from 98.1% at sea level to 90.8% at 10,000 feet. The LFIA produced rapid, visually interpretable results within minutes. The LFIA demonstrated high sensitivity and specificity for detecting hypoxia-induced HIF-1α in saliva, potentially as a rapid point-of-care tool for hypoxia monitoring in aviation and extreme environments.

Keywords: Atmospheric pressure; General aviation aircraft; High altitude; Physiological responses.

INTRODUCTION

Aircrew exposed to high-altitude environments for prolonged durations are at increased risk of adverse physiological and operational consequences, posing significant concerns for both human health and aircraft safety. Altitude-related environmental stressors remain a critical issue in aviation and aerospace medicine, particularly in military and high-performance flight operations (Ji *et al.* 2021). For safety reasons, adequate acclimatization is essential for individuals ascending to high altitudes. Although the physiological effects of altitude are most pronounced above 8,000 feet (2,438 m), measurable changes can occur at elevations as low as 5,000 feet (1,524 m).

Among the various environmental changes encountered at altitude – including reductions in ambient temperature and humidity – the defining feature is the reduction in barometric pressure. This reduction leads to a decrease in the partial pressure of oxygen (PO_2) throughout the oxygen transport cascade, from inspired air to the alveoli, arterial blood, tissues, and ultimately venous circulation (Kim *et al.* 2015). The magnitude of this decline in PO_2 increases with altitude and duration of exposure, particularly

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during extended high-altitude flight or space missions. These reductions in oxygen availability trigger a range of compensatory physiological responses over minutes to weeks, enabling partial adaptation to hypoxic environments. Acute altitude exposure influences vascular tone in systemic resistance vessels and increases ventilation and sympathetic nervous system activity through peripheral chemoreceptor activation (Kim *et al.* 2015).

Ascent to high altitude induces complex physiological adaptations aimed at counteracting hypobaric hypoxia by enhancing oxygen delivery and modifying tissue oxygen utilization through metabolic regulation. At the cellular level, these adaptive responses are primarily regulated by the hypoxia-inducible factor (HIF) signaling pathway. Activation of this pathway promotes glycolytic metabolism while suppressing oxidative phosphorylation under low-oxygen conditions (O'Farrell 2008). Hypoxia-inducible factor-1 (HIF-1), particularly its oxygen-sensitive alpha subunit (HIF-1α), plays a central role in maintaining oxygen homeostasis by facilitating cellular adaptation to hypoxia during acclimatization, hypoxemia, and inflammatory responses. HIF-1α is ubiquitously expressed across tissues and is rapidly degraded under normoxic conditions via oxygen-dependent hydroxylation; however, under hypoxic conditions, it stabilizes and accumulates, making it a reliable molecular marker of cellular hypoxia (O'Farrell 2008).

The operational relevance of hypoxia detection in aviation is underscored by retrospective analyses of military aircraft accidents. Studies conducted after the Second World War documented numerous unexplained aviation incidents suspected to be linked to hypoxia. (Konecci 1957; Lewis 1948; McBurney 1974). Identification of hypoxia exposure during postmortem investigations remains critical for flight safety analysis. A retrospective study by Tripathi *et al.*, covering the period 1986-1995, involving Army Aviation helicopter operations at high altitude, reported 29 accidents, with hypoxia implicated in approximately 24% of cases (Tripathi *et al.* 1996). Furthermore, pilot incapacitation due to hypoxia was confirmed as the cause of the Indian Air Force MiG-29 crash at Sirsi, Karnataka, on 11 April 2002. These findings emphasize the persistent risk of undetected hypoxia in aviation operations (Indian Air Force 2002).

Conventionally, hypoxia assessment has relied on physiological, biochemical, and molecular approaches. Physiological methods such as arterial blood gas analysis, pulse oximetry, and tissue oxygen tension measurements provide indirect or localized information on oxygen availability but are limited by invasiveness, lack of cellular specificity, and inability to reflect intracellular hypoxic responses (Andryukov 2020). At the molecular level, hypoxia is commonly evaluated through the detection of hypoxia-responsive markers, including HIF-1α, carbonic anhydrase IX (CAIX), and glucose transporter-1 (GLUT-1) (Brown *et al.* 2013). Conventional analytical techniques for HIF-1α detection include Western blotting, enzyme-linked immunosorbent assay (ELISA), immunohistochemistry, quantitative polymerase chain reaction, and mass spectrometry-based proteomics (Files *et al.* 2005; Ghosh and Pant *et al.* 2010). While these methods provide high sensitivity and specificity, they are generally time-consuming, labor-intensive, and require sophisticated laboratory infrastructure and skilled personnel (Ghosh and Pant *et al.* 2010). Moreover, many of these techniques are not suitable for rapid or point-of-care hypoxia assessment, limiting their translational and clinical applicability. Consequently, there remains a significant need for simple, rapid, and cost-effective methods to detect hypoxia-associated biomarkers.

HIF-1α is widely recognized as a master regulator of cellular responses to hypoxia, governing the transcription of genes involved in angiogenesis, metabolism, and cell survival. Due to its rapid stabilization under low-oxygen conditions, HIF-1α has been extensively studied as a reliable molecular biomarker for hypoxia. Previous studies have predominantly utilized laboratory-based techniques such as ELISA, Western blotting, and immunohistochemistry to quantify HIF-1α expression. Although these approaches demonstrate high analytical accuracy, they typically require prolonged processing times and are unsuitable for real-time or field-based applications.

In recent years, efforts have been made to develop rapid detection platforms for hypoxia biomarkers, including electrochemical biosensors, fluorescence-based assays, and microfluidic systems (Koczula and Gallotta 2016; Li *et al.* 2022). However, many of these technologies still depend on complex instrumentation or extensive sample preparation (Shaharuddin *et al.* 2023a). Lateral flow immunoassays (LFIAs), in contrast, offer distinct advantages such as rapid turnaround time, ease of use, portability, and minimal sample handling (Koczula and Gallotta 2016; Li *et al.* 2022). Despite their widespread application in infectious disease diagnostics and protein detection, studies employing LFIA for the rapid detection of HIF-1α remain limited (Shaharuddin *et al.* 2023b). Furthermore, comparative evaluations between LFIA-based platforms and conventional analytical methods for HIF-1α detection are scarce.

Despite the recognized importance of hypoxia monitoring, current detection methods rely largely on physiological measurements or laboratory-based molecular assays that are not suited for rapid or field deployment. Therefore, there is a critical need for a simple, rapid, and non-invasive diagnostic approach capable of detecting early hypoxic changes. In this study, the feasibility of an LFIA for the detection of salivary HIF-1α as a biomarker of hypoxia following simulated altitude exposure was investigated. This approach aims to provide a practical point-of-care tool for early hypoxia detection in aviation, military, and extreme environmental settings.

METHODS

Materials

Nitrocellulose (NC) membranes (pore size 15-20 μm), sample pads, conjugate pads, and absorbent pads were obtained from commercial LFIA component suppliers. Anti-HIF-1α monoclonal antibodies (capture and detection antibodies) and recombinant human HIF-1α protein were purchased from certified biochemical suppliers. Colloidal gold nanoparticles (20-40 nm) were used as the signal reporter. Bovine serum albumin (BSA), phosphate-buffered saline (PBS), Tween-20, and other analytical-grade reagents were obtained from standard laboratory vendors. Saliva collection tubes were sterile and RNase/DNase-free.

Development of the LFIA

Dot blot enzyme immunoassay

Nitrocellulose membranes were cut into appropriate sizes and marked with a 1 × 1 cm grid. Membranes were pre-wetted in blotting buffer for 5 minutes and placed on wet filter paper. Using a narrow-mouth pipette, each sample was spotted in the center of each grid square. After drying, non-specific sites were blocked with 5% BSA in PBS-Tween 20 (PBS-T) for 1 hour at room temperature. Membranes were then dried at 37 °C for 30 minutes. For detection, an anti-HIF-1α monoclonal antibody conjugated to horseradish peroxidase at a 1:500 dilution in 5% BSA was applied to each spot and incubated for 2 hours at room temperature under moist conditions. Positive controls produced distinct dark-brown spots, confirming successful antigen-antibody binding (Fig. 1).

Source: Elaborated by the authors.

Figure 1. Optimization of dot blot analysis.

Reagents and materials

The LFIA strip consists of four functional components assembled on an adhesive backing card: a sample pad, a conjugate pad, an NC membrane, and an absorbent pad. Monoclonal anti-HIF-1α antibodies were used as both detection and capture antibodies. Colloidal gold nanoparticles served as the colorimetric signal reporters. Bovine serum albumin (BSA), PBS, and Tween-20 were used as blocking and washing agents to minimize nonspecific binding.

Preparation of gold-antibody conjugates

Colloidal gold nanoparticles were conjugated with anti-HIF-1α detection antibodies using passive adsorption. Briefly, the pH of the gold nanoparticle solution was adjusted to optimal binding conditions, followed by the addition of anti-HIF-1α antibody at a predetermined concentration. The mixture was incubated at room temperature with gentle agitation to allow antibody adsorption onto the nanoparticle surface. Unbound antibodies were blocked using BSA, and the conjugates were purified by centrifugation and resuspended in conjugate storage buffer. The prepared gold-antibody conjugates were stored at 4 °C until use.

Preparation of the conjugate pad

The conjugate pad was pre-treated with buffer containing stabilizing agents and dried at a controlled temperature. The gold-antibody conjugate solution was uniformly dispensed onto the conjugate pad and dried under desiccated conditions. Prepared conjugate pads were stored in sealed containers with desiccant prior to assembly.

Immobilization of capture reagents on the NC membrane

Anti-HIF-1α capture antibodies were immobilized onto the NC membrane to form the **test line**, while anti-species secondary antibodies were immobilized downstream to form the **control line**. Antibodies were dispensed using an automated or semi-automated dispenser at defined concentrations. After dispensing, membranes were dried at room temperature and blocked to minimize non-specific binding.

Assembly of the LFIA test strip

The LFIA strip was assembled by sequentially overlapping the sample pad, conjugate pad, NC membrane, and absorbent pad on an adhesive backing card, ensuring appropriate overlap for uninterrupted capillary flow. The assembled cards were cut into individual strips and stored in airtight packaging with desiccant until use (Fig. 2).

Source: Elaborated by the authors.

Figure 2. Photo of the housing used in this method to demonstrate how compressing the lateral flow strip improves reliability. The actual size of the device is comparable to the SARS-CoV-2 detection kits.

Saliva sample collection and handling

Unstimulated saliva samples were collected from study subjects to minimize dilution effects. Participants were instructed to refrain from eating, drinking, smoking, or performing oral hygiene procedures for at least 30 minutes prior to collection. Saliva samples were collected during a consistent time period to reduce circadian variability. Samples were collected into sterile tubes and immediately placed on ice. Following collection, saliva samples were centrifuged to remove debris and mucins. The supernatant was aliquoted and either analyzed immediately or stored at -20 °C until analysis. Repeated freeze-thaw cycles were avoided to preserve protein integrity.

Detection mechanism of the lateral flow immunoassay (LFIA)

Principle of the LFIA detection mechanism

The LFIA developed in this study operates on a sandwich immunoassay principle for the qualitative detection of HIF-1α in human saliva. The assay utilizes AuNP-labeled monoclonal antibodies as visual signal reporters and immobilized capture antibodies on an NC membrane to enable rapid, colorimetric detection.

Upon application of the saliva sample to the sample pad, capillary forces drive the sample through the conjugate pad, where, if HIF-1α is present, it binds to the AuNP-conjugated anti-HIF-1α detection antibodies. This antigen-antibody complex then migrates along the NC membrane toward the test and control regions. The accumulation of gold nanoparticles at the test line produces a visible red-colored band, indicating a positive result.

Lateral flow immunoassay (LFIA) detection procedure

For analysis, a defined volume of saliva sample or standard solution was applied to the sample pad of the LFIA strip. The test was allowed to develop at room temperature. Migration of the sample and formation of visible lines were monitored visually. HIF-1α detection was evaluated using serial concentrations of recombinant HIF-1α to establish assay performance.

Each concentration was tested in multiple replicates. The appearance of the test and control lines was recorded at a predefined detection time. The test was considered valid when the control line appeared, indicating proper sample migration. Absence of the control line was classified as an invalid result.

Interpretation of results

The LFIA provided qualitative detection based on visual interpretation of colored line formation. The intensity of the test line increased with increasing HIF-1α concentration; however, for this study, results were interpreted as positive or negative relative to a visually defined detection threshold. Independent observers evaluated the test results to minimize subjective bias.

Study subjects

This study is a prospective cohort study. The 24 samples of fit individuals were recruited from the Institute of Aviation Medicine. The subjects were divided into two groups which included the first group, a control group of 12 samples taken at sea level, and the second group of 12 samples taken at an altitude of 10,000 feet, using stratified sampling to ensure both groups were comparable on relevant variables such as fitness level, age, and previous altitude experience. Written consent was obtained from the subjects who fulfilled the inclusion criteria. Saliva for both groups was tested using a rapid colorimetric test, along with measurement using an SpO_2 monitor (fingertip pulse oximeter). Saliva was chosen for its non-invasiveness. The study protocol was approved by the Institutional Ethics Committee (approval no. 25/2024, dated December 18, 2024).

Statistical analysis

All statistical analyses were performed using SPSS software (version 25.0; IBM Corp., USA). Descriptive statistics were calculated for demographic and baseline variables and are presented as mean ± standard deviation. Differences in SpO_2 levels between the sea-level (control) condition and simulated high-altitude exposure were assessed using independent samples *t*-tests. The association between hypoxic exposure and the presence of salivary HIF-1α detected by LFIA was evaluated by comparing test outcomes across the two experimental conditions. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Dot plot

To further investigate the feasibility of HIF-1α as a protein marker for hypoxia studies, saliva samples were examined using dot blot analysis. A distinct brown-colored precipitate was formed at the protein-spotted regions, indicating successful antigen-antibody binding. The intensity of the color developed increased proportionally with the concentration of HIF-1α immobilized on the membrane, demonstrating concentration-dependent signal generation. Figure 3 displays representative dot blot patterns of saliva samples taken from individuals. Higher protein concentrations produced darker and more localized spots (exposed to high altitude), while lower concentrations resulted in fainter signals (at sea level). The spotted areas remained well defined with minimal diffusion, suggesting strong affinity of HIF-1α to the NC membrane and effective immobilization during deposition. These results confirm that the dot blot assay successfully detected HIF-1α and provided a reliable preliminary platform for validating antibody-antigen interactions prior to LFIA development (Fig. 3).

Source: Elaborated by the authors.

Figure 3. Detection of HIF-1α in saliva samples. C = control (sea level); S = sample (high altitude, 10,000 feet).

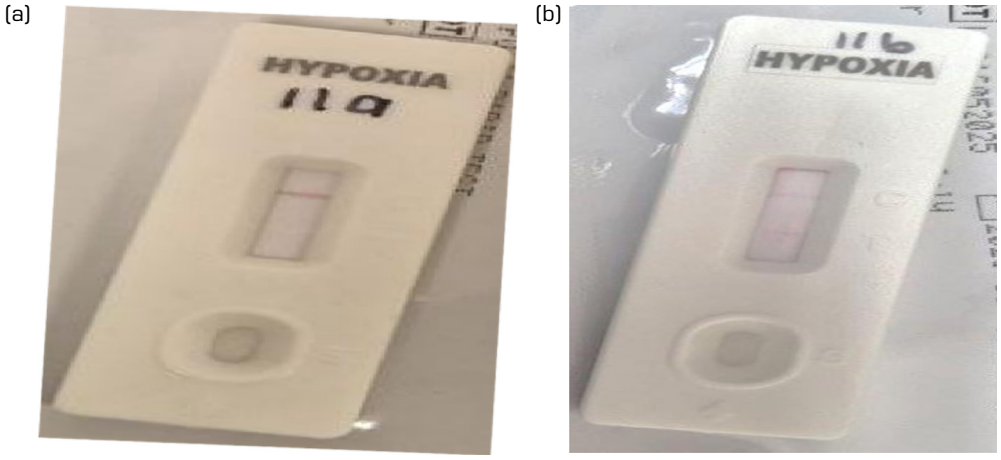
Participant characteristics

A total of 24 participants were included in the study. The mean age of the participants was 34.3 ± 5.9 years, indicating a young- to middle-aged adult population. The cohort was predominantly male. The mean body mass index (BMI) was 24.7 ± 3.7 kg/m², with values ranging from underweight to obese, indicating heterogeneity in body composition among participants. The majority of the participants were not receiving any pharmacological treatment at the time of the study. Regarding lifestyle factors, three participants (25%) were smokers, while four participants (33.3%) reported vaping. Most participants were non-smokers and non-vape users.

Colorimetric detection of HIF-1α

Biomarker of hypoxia (HIF-1) expression using a colorimetric test is shown in Fig. 4. In this design, assay interpretation was based on the visual appearance of red-colored lines at the test and control regions. A sample was considered negative when a red signal appeared only at the control line and there was an absence of a visible test line, indicating proper fluid migration and assay validity, as shown in Fig. 4a. A sample was considered positive when red signals were observed at both the test and control lines, confirming the presence of HIF-1α protein, as shown in Fig. 4b.

Baseline (sea level) shows that 12 participants tested negative for HIF-1α in saliva using the colorimetric LFIA strips, while post-exposure (hypobaric chamber, simulated 10,000 feet) shows that all 12 participants tested positive for HIF-1α, indicating hypoxia-induced upregulation of the biomarker. This showed a 100% conversion rate from negative to positive after exposure to simulated altitude, confirming the test's sensitivity in detecting early hypoxia show in Fig. 4.



Source: Elaborated by the authors.

Figure 4. Results of the colorimetric test of HIF-1α. (a) at sea level (pre-test); (b) at high altitude (post-test).

Oxygen saturation results

Oxygen saturation levels (SpO_2) decreased significantly following simulated altitude exposure. At sea level, the mean SpO_2 was 98.1 ± 0.67, whereas exposure to a simulated altitude of 10,000 feet resulted in a marked reduction to 90.8 ± 0.72. Statistical analysis revealed a highly significant difference between the two conditions ($p < 0.001$), confirming the induction of hypoxic stress under simulated altitude exposure (Table 1).

Values are presented as individual measurements and group means. SpO_2 was measured by fingertip pulse oximetry. Sea-level measurements represent pre-exposure values; 10,000 ft measurements represent post-exposure values following hypobaric chamber simulation.

Table 1. Mean SpO_2 values measured at sea level (pre-test) and after simulated altitude exposure at 10,000 feet (post-test).

Subject no.	SpO_2 at sea level (%)	SpO_2 at 10,000 ft (%)
1	98	90
2	99	91
3	98	90
4	97	91
5	98	92
6	99	90
7	98	90
8	99	91
9	98	92
10	98	90
11	98	91
12	97	90
13	98	90
14	99	91
15	98	90
16	97	91
17	98	92
18	99	90
19	98	90
20	99	91
21	98	92
22	98	90
23	98	91
24	97	90
Mean ± SD	98.08 ± 0.67	90.67 ± 0.72

Source: Elaborated by the authors.

Association between hypoxic exposure and salivary HIF-1α detection by LFIA

The association between hypoxic exposure and salivary HIF-1α detection was assessed by comparing LFIA test outcomes between the sea level (control) and simulated high-altitude conditions. The analysis revealed a significant drop in SpO_2 levels ($t = 19.2$, $p < 0.001$), and this corresponded with the appearance of the HIF-1α signal on the LFIA strips in the altitude group. The difference in SpO_2 levels between the two groups was statistically significant ($p < 0.001$), confirming the physiological effect of hypobaric hypoxia at 10,000 feet. Furthermore, the qualitative data from the colorimetric test were 100% concordant with the drop in SpO_2 levels, supporting the utility of HIF-1α detection as a reliable hypoxia biomarker. Every participant experienced a drop in SpO_2, consistent with physiological expectations under hypobaric conditions.

Hypoxia symptoms

Despite measurable decreases in SpO_2 and biomarker positivity, none of the participants reported subjective symptoms of hypoxia, such as headache, dizziness, or confusion. This supports the potential of the HIF-1α biomarker as a pre-symptomatic marker of hypoxia.

DISCUSSION

Optimization of antibody-gold conjugation and capture reagent immobilization

The performance of an LFIA is highly dependent on the optimal concentration of antibody–gold conjugates and the amount of immobilized capture antibody on the NC membrane. In this study, the concentration of anti-HIF-1α antibody used for gold nanoparticle conjugation was optimized to achieve a balance between signal intensity and background noise. Insufficient antibody loading resulted in weak or inconsistent test line formation, likely due to limited antigen–antibody interactions, whereas excessive antibody concentrations led to nanoparticle aggregation and reduced capillary flow, adversely affecting assay reproducibility (Pham *et al.* 2021).

Similarly, the immobilization density of the capture antibody on the test line played a critical role in assay sensitivity. Lower immobilization concentrations produced faint or undetectable test lines, particularly at low HIF-1α concentrations, while excessively high capture antibody loading increased nonspecific binding and background coloration. The optimized capture antibody concentration provided a clearly distinguishable test line with minimal background interference, demonstrating the importance of fine-tuning both conjugate and capture reagent parameters to ensure reliable LFIA performance (Pohanka 2021).

Detection performance and cut-off determination for HIF-1α

The LFIA developed in this study demonstrated a concentration-dependent color development corresponding to HIF-1α levels. A visible test line was observed starting from the lowest detectable concentration, indicating effective antigen recognition and signal amplification through gold nanoparticle labeling. At lower HIF-1α concentrations, a faint test line was observed, which progressively intensified with increasing analyte concentration (Koczula and Gallotta 2016).

The cut-off value was defined as the lowest concentration at which the test line could be consistently distinguished from the negative control by visual inspection across repeated experiments. Concentrations below this threshold did not produce a discernible test line, indicating the limit of visual detection for the assay. The ability to detect faint yet reproducible color development at low HIF-1α concentrations highlights the sensitivity of the LFIA and supports its potential utility for rapid hypoxia screening. Importantly, the appearance of the control line in all valid tests confirmed proper assay function and fluid migration (Shaharuddin *et al.* 2023a).

Selectivity and cross-reactivity assessment

High selectivity is essential for reliable biomarker detection, particularly when analyzing complex biological matrices such as saliva. In this study, the selectivity of the LFIA was evaluated by exposing the test strips to non-target proteins and potential interfering components commonly present in saliva. These analytes did not produce a visible test line, while the control line remained clearly visible, confirming correct assay operation (Yang *et al.* 2017).

The absence of test line formation in the presence of non-specific proteins provides strong evidence of minimal cross-reactivity and demonstrates that the observed signal is attributable specifically to HIF-1α binding. This selectivity can be attributed to the use of highly specific monoclonal antibodies and the optimized assay conditions that reduce nonspecific interactions. Collectively, these findings confirm that the LFIA exhibits high specificity for HIF-1α and is suitable for selective hypoxia biomarker detection in saliva samples (Shin *et al.* 2021).

The optimized antibody-gold conjugation, carefully controlled capture antibody immobilization, and demonstrated detection sensitivity and selectivity collectively validate the robustness of the developed LFIA. The ability to rapidly detect HIF-1α with minimal cross-reactivity underscores the potential of this platform as a practical and reliable tool for hypoxia assessment, particularly in settings where rapid and user-friendly diagnostics are required (Formenti *et al.* 2010).

This study successfully demonstrated the feasibility, specificity, and sensitivity of a rapid colorimetric LFIA sensor for detecting HIF-1α expression following simulated high-altitude exposure. The complete absence of HIF-1α at sea level and its consistent detection at 10,000 feet confirm the sensor's high diagnostic accuracy in differentiating normoxic and hypoxic conditions. These results validate the potential of LFIA-based HIF-1α biomarker detection as a rapid, non-invasive, and reliable approach for monitoring early hypoxia. Further studies involving larger sample sizes, real-flight validation, and extended exposure durations are warranted to confirm the device's clinical robustness and field applicability.

CONCLUSION

The developed hypoxia detection device presents a novel and practical solution for monitoring health in real time. Its ability to rapidly and reliably detect early biomarkers of hypoxia holds great potential for broader applications in aerospace medicine, emergency response, and remote health monitoring. Further research is warranted to explore the long-term effects of hypoxia and to validate the clinical utility of LFIA-based biomarker detection in diverse operational settings.

CONFLICTS OF INTEREST

Nothing to declare.

AUTHOR CONTRIBUTIONS

Conceptualization: Shaharuddin S, Hassan HA, and Miskan M; **Methodology:** Shaharuddin S and Mohammad Z; **Formal analysis:** Mohd S Hassan HA; **Resources:** Mohd S and Hashim R; **Data Curation:** Miskan M and Hashim R; **Writing - Original Draft:** Shaharuddin S and Hashim R; **Writing - Review & Editing:** Miskan M and Hassan HA; **Visualization:** Nordin MKN and Zainal NS; **Supervision:** Mohammad Z and Shaharuddin S; **Funding acquisition:** Shaharuddin S; **Final approval:** Shaharuddin S.

DATA AVAILABILITY STATEMENT

The data will be available upon request.

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DECLARATION OF USE OF ARTIFICIAL INTELLIGENCE TOOLS

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