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Evaluation of the Co-Exposure of Acute Restraint Stress and Rem Sleep Deprivation on Liver Function in Female Wistar Rats

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Authors' contributions

This work was carried out in collaboration among all authors. Authors GOO, OAL, HOA, FYO and TAJ did methodology, formal analysis, data curation, writing of original draft, review, and editing, conceptualization, validation, visualization, editing of final draft, and supervision, final review and preparation of the manuscript for publication. The authors declare that all data were generated in-house and that no paper mill was used. All authors read and approved the final manuscript.

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ABSTRACT

Co-exposure to psychological and physiological stressors, such as acute restraint stress and REM sleep deprivation, may have harmful effects on hepatic integrity that are synergistic. This study investigated the effects of combined acute restraint stress and REM sleep deprivation on the liver function of female Wistar rats.

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Twenty-four female Wistar rats (n=6) were divided randomly into four groups: control, restraint stress only (RSS; 30 minutes/day), sleep deprivation (SDD; six hours every 48 hours), and restraint stress + sleep deprivation (RSD). After 28 days, rats were sacrificed and key markers of hepatic function were measured in serum, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT) total protein, globulin and albumin.

REM sleep deprivation alone (SDD) significantly increased ($p<0.05$) serum levels of AST, ALT, ALP, and GGT compared to control. Restraint stress + sleep deprivation (RSD) showed a further significant increase ($p<0.01$) in AST and ALT compared to both control and RSS groups. Total protein was significantly decreased ($p<0.05$) in the SDD and RSD groups. No significant changes were observed in albumin or globulin levels across the groups.

The study concludes that REM sleep deprivation is a potent inducer of hepatic injury, and co-exposure with acute restraint stress has a synergistic effect, exacerbating hepatocellular damage and impairing the liver's synthetic function. This demonstrates the need of managing interrelated stress and sleep disruption for maintaining liver health.

Keywords: Restraint stress; REM sleep deprivation; liver function; hepatotoxicity; oxidative stress; wistar rats.

1. INTRODUCTION

Liver dysfunction induced by psychological and physiological stressors represent is an emerging health concern, particularly in the context of modern lifestyles characterized by chronic stress and sleep disruption (Demori & Grasselli, 2023). Acute restraint stress is a well-established model of psychological stress that elicits a robust neuroendocrine response, primarily through the activation of the hypothalamic-pituitary-adrenal (HPA) axis (Figliomeni, 2020). Concurrently, rapid eye movement sleep deprivation is a potent physiological stressor known to disrupt metabolic homeostasis and exacerbate stress-induced pathologies (Thondala *et al.,* 2024). The co-exposure to these multifactorial stressors may have synergistic detrimental effects on hepatic integrity and function, though the precise mechanisms remain to be fully elucidated.

As a central metabolic organ, the liver is highly susceptible to stress hormones and oxidative damage (Chainy and Sahoo, 2020). Studies indicate acute restraint stress caused hepatic injury, as indicated by alteration in standard liver function tests (Oh *et al.,* 2020). This injury manifests through the disruption of hepatocellular membrane integrity, leading to the leakage of enzymes and impairment of the liver's synthetic and detoxification capacities (Mihajlovic & Vinken, 2022). Oxidative stress has been documented to disrupt hepatocellular membrane integrity, promote lipid peroxidation, and induce inflammation (Yang *et al.,* 2022). Based on previous studies, acute restraint stress and rapid eye movement sleep deprivation independently produce copious amounts of reactive oxygen species (ROS) which induce hepatic damage via two mechanisms: direct action on hepatocellular components such as DNA, proteins, and lipids (Wang *et al.,* 2021) and indirect action via the activation of inflammatory cascades and stress-signaling pathways (Meng *et al.,* 2022). In addition to this, stress-induced hepatocyte damage has been reported to increase the permeability of liver cell membranes, resulting in the release of cytosolic enzymes such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) into circulation, which serve as primary indicators of hepatic damage (Conde *et al.,* 2022; Vairetti et al., 2021).

The paradigm of co-exposure is particularly crucial, as physiological and psychological stressors can interact in a synergistic manner, leading to results more severe than the sum of their separate impacts. Recent research demonstrated that co-exposure to sleep fragmentation and social defeat stress in mice synergistically exacerbated hepatic inflammation and markers of injury, beyond the effects of either stressor alone (Gao *et al.,* 2024). During co-exposure to acute restraint stress and rapid eye movement sleep deprivation, reactive oxygen species (ROS) production causing hepatic dysfunction is precipitated through multiple synergistic pathways. Acute restraint stress rapidly elevates circulating catecholamines and glucocorticoids (corticosterone in rats), which can directly impact hepatic metabolism and induce metabolic shifts that increase ROS production in the liver via mitochondrial dysfunction and NADPH oxidase

activation that compromise liver function (Dantzer *et al.,* 2021; Fan *et al.,* 2023). Furthermore, glucocorticoids can exacerbate hepatic dysfunction through dual mechanistic pathways: firstly, by impairing cellular antioxidant defense systems through the downregulation of key enzymes such as superoxide dismutase and glutathione peroxidase (Mooli *et al.,* 2022; Allameh *et al.,* 2023), and secondly, by altering hepatic metabolic function through the modulation of gluconeogenic and glycolytic pathways while simultaneously impairing the organ's detoxification capacity via suppression of cytochrome P450 enzyme activity (Esteves *et al.,* 2021; Więckowska *et al.,* 2023). Rapid eye movement sleep deprivation, on the other hand, induces ROS generation via sustained sympathetic overactivity, metabolic overload, and disruption of circadian redox rhythms (Richardson & Mailloux, 2023). This generated ROS is capable of forming peroxynitrite radicals which can interfere with cellular structures such as proteins, lipids, and DNA to cause severe oxidative damage to the liver (Jomova et al*.,* 2023). This metabolic disruption is capable of altering the structural and functional integrity of hepatocytes, leading to impaired biliary function and changes in biomarkers such as alkaline phosphatase (ALP) and bilirubin (Tamber *et al.,* 2023). The combined stress exposure is also capable of disrupting the liver's synthetic function, potentially leading to alterations in serum protein and albumin levels, which serve as critical indicators of the liver's biosynthetic capacity (Conde *et al.,* 2022; Sadasivam *et al.,* 2022).

Based on the multifactorial nature of the pathway involved in the co-exposure of acute restraint stress and rapid eye movement sleep deprivation (Pan, 2022), a detailed evaluation is required to understand the synergistic impact on clinical liver function parameters. This study therefore investigates the effect of the co-exposure of acute restraint stress and REM sleep deprivation on standard liver function tests, including serum levels of ALT, AST, ALP, globulin, total protein, and albumin in female Wistar rats.

2. MATERIALS AND METHODS

2.1 Experimental Animals

A total of twenty-four (24) female Wistar rats (weighing 180–220 g) were purchased from a commercial breeder in Ogbomosho, Nigeria. The animals were acclimatized for two weeks before the commencement of the experiment, and kept throughout the experiment in a well-ventilated plastic cages in the Animal House (temperature 28-31°C; photoperiod:12-h natural light and 12-h dark; humidity:50-55 %) of Faculty of Basic Medical Sciences (FBMS), Ladoke Akintola University of Technology (LAUTECH) with free access to fed and water *ad libitum*. The animal handling procedure was done according to the guidelines for the use and care of laboratory animals, as recommended by the animal care and use research ethic committee of LAUTECH, were followed.

2.2 Experimental Design

After acclimatization, Twenty-four (24) male Wistar rats were divided into 4 groups (n=6) as follows:

Group 1: Control (CTL): Received no stress induction, only standard housing with feed and water *ad libitum* for 28 days.

Group 2: Restraint Stress Only (RSS): Subjected to restraint stress for 30 minutes daily for 28 days.

Group 3: Sleep Deprivation Only (SDD): Subjected to intermittent REM sleep deprivation for 6 hours every 48 hours for 28 days.

Group 4: Restraint Stress + Sleep Deprivation (RSD): Subjected to both restraint stress (30 minutes daily) and intermittent REM sleep deprivation (6 hours every 48 hours) for 28 days.

2.3 Induction of Experimental Model

2.3.1 Restraint stress procedure

Restraint stress was induced according to the method described by Owolabi et al. (2024). Rats were individually placed inside a wire mesh restrainer that limited their movement without inflicting pain, for a duration of 30 minutes each day. After the procedure, they were returned to their home cages.

2.3.2 REM sleep deprivation procedure

Rapid Eye Movement (REM) sleep deprivation was achieved using the inverted flower pot (multiple platform) method, as described by Nollet et al. (2020). Rats were placed on small platforms (diameter: 10.6 cm, height: 11.3 cm) within a water-filled tank (diameter: 98 cm,

height: 46 cm) divided into three compartments. The water level was maintained just below the platform surface. This setup allows for non-REM sleep but induces awakening by a fall into the water when muscle atonia occurs during REM sleep. The procedure was conducted for 6 hours intermittently (every 48 hours). The top of the tank was covered with a wire mesh to prevent escape.

2.4 Euthanasia and Blood Collection

After 28 days of treatment and a 24-hour post-exposure period, all animals were sacrificed. Rats were euthanized using a chemical method with chloroform inhalation. Animals were placed in a desiccator containing cotton wool soaked with approximately 2ml of chloroform. Unconsciousness was achieved within 1-2 minutes, after which cardiac puncture was performed to collect blood samples. Blood was transferred into Ethylene Diamine Tetra-acetic Acid (EDTA) bottles for plasma separation.

2.5 Biochemical Analysis

Serum biochemical parameters were analyzed using commercial reagent kits on a spectrophotometer (BM 5010, Magdeburg, Germany). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), albumin (ALB), and total protein (TP) were determined using kits purchased from Human GmbH (Magdeburg, Germany). Alkaline phosphatase (ALP) was assessed using a kit from Spinreact (Barcelona, Spain). All assays were performed in duplicate according to the manufacturers' protocols without modification. The specific wavelengths for each assay were as follows: ALT and AST at 340 nm, ALP and GGT at 405 nm, and albumin and total protein at 546 nm. Globulin (GLO) concentration was calculated indirectly using the formula: Globulin (g/dL) = Total Protein (g/dL) - Albumin (g/dL). Assay performance was verified for each parameter by analyzing manufacturer-provided quality control materials (normal and pathological levels) prior to running experimental samples.

2.6 Statistical Analysis

The study's numerical data were expressed as mean ± standard error of mean (Mean ± SEM). A one-way Analysis of variance (ANOVA) with Graph Pad Prism version 7.0 (Graph Pad statistical software, Inc., USA) was used to compare within groups and Tukey's Post-hoc test was used for multiple comparison. $p<0.05$ was considered statistically significant.

3. RESULTS

Based on the result obtained, serum levels of liver function enzymes presented in Table 1 revealed AST and ALT was significantly increased ($p<0.05$) in the SD group when compared to control, while a further significant increase ($p<0.01$) was observed in the RS+SD group compared with both the control and RS groups. ALT and AST activities showed no difference between the control and RS groups, but was significantly increased ($p<0.05$) in the SD group. ALP levels were slightly reduced in the RS group compared with control but were not statistically significant. In contrast, ALP was significantly increased ($p<0.05$) in the SD group, and the RS+SD group showed a marked elevation ($p<0.05$) compared to control. GGT activity was increased ($p<0.05$) in SD rats compared to control, with further elevation observed in the RS+SD group ($p<0.05$) when compared with control and RS groups.

Total protein were significantly decrease ($p<0.05$) in both SD and RS+SD when compared to control while there is no significant difference in RS when compared to control. Albumin and globulin showed no significant difference in RS, SD and RS+SD when compared to control.

Table 1. Effects of co-exposure of acute Restraint stress and REM sleep deprivation on serum levels of liver function enzymes in female Wistar rats

Liver Enzymes	Control	RS	SD	RS + SD
AST (U/L)	52.32 ± 2.87	57.47 ± 2.25	61.79 ± 4.96a	79.89 ± 5.49ab
ALT (U/L)	27.40 ± 1.47	27.28 ± 3.56	39.77 ± 5.07a	47.51 ± 5.47ab
ALP (U/L)	36.95 ± 4.51	33.42 ± 2.85	42.87 ± 3.40	57.62 ± 6.12a
GGT (U/L)	13.01 ± 1.08	16.85 ± 1.90	20.52 ± 2.95a	22.77 ± 2.00a

Data were represented as mean ± SEM., n=5. P< 0.05 was considered as statistically significant.

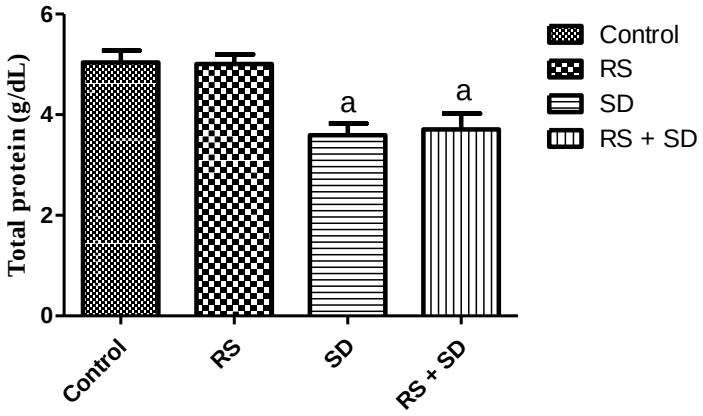

Fig. 1. Effects of co-exposure of acute Restraint stress and REM sleep deprivation on total protein in female Wistar rats

Data were represented as mean ± SEM., n=6. P< 0.05 was considered as statistically significant.

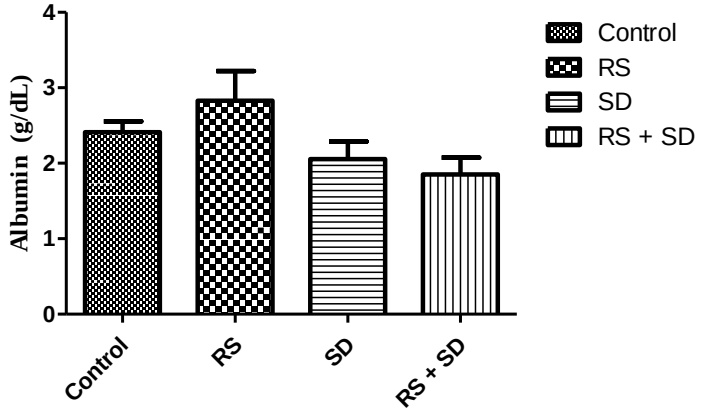

Fig. 2. Effects of co-exposure of acute Restraint stress and REM sleep deprivation on albumin in female Wistar rats

Data were represented as mean ± SEM., n=6. P< 0.05 was considered as statistically significant.

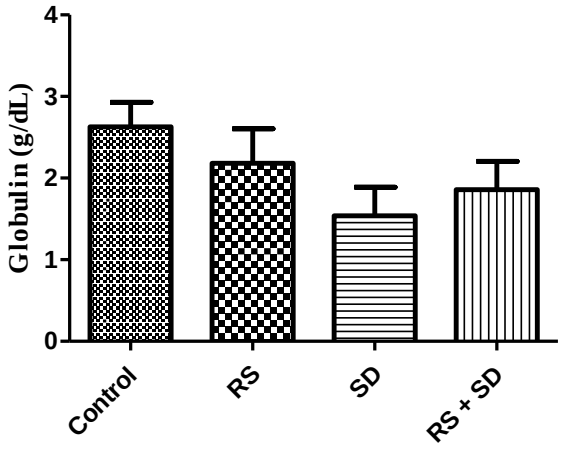

Fig. 3. Effects of co-exposure of acute Restraint stress and REM sleep deprivation on globulin in female Wistar rats

Data were represented as mean ± SEM., n=6. P< 0.05 was considered as statistically significant.

4. DISCUSSION

This study shows that acute restraint stress and REM sleep deprivation, both individually and particularly in combination, can induce significant hepatic injury in female Wistar rats, as evidenced by the impairment of liver function enzymes and protein synthesis. This study also demonstrates the exacerbating effect of the co-exposure to these stressors, highlighting a potential synergistic effect on liver pathophysiology.

Liver function enzymes investigated include aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and gamma-glutamyl transferase (GGT). In this study, the observed increase in the serum levels of these enzymes in the SD and RS+SD groups is a primary indicator of hepatocyte damage and compromised cellular membrane integrity. This is in agreement with the findings of (Costa *et al.,* 2016; Kumar *et al.,* 2021) that reported elevated liver enzymes following sleep deprivation. These enzymes are normally contained within the cytoplasm of hepatocytes; their leakage into the bloodstream is a direct consequence of cellular injury (Hirao *et al.,* 2022). ALT is considered a more specific marker for hepatic parenchymal injury, while increases in ALP and GGT can indicate cholestatic stress or damage to the biliary tract (Tamber *et al.,* 2023).

The significant elevation of these enzymes in the SD group suggests that REM sleep deprivation is a potent inducer of hepatic stress. Sleep is crucial for metabolic homeostasis and cellular repair (Morrison *et al.,* 2022). Disruption of sleep architecture, particularly REM sleep, can lead to increased oxidative stress and inflammatory responses (Maniaci *et al.,* 2021). The further significant increase in AST, ALT, and GGT in the RS+SD group compared to SD alone indicates a synergistic hepatotoxic effect when sleep deprivation is combined with psychological stress. This is consistent with studies showing that restraint stress can amplify pro-inflammatory pathways and oxidative damage in the liver (Madrigal *et al.,* 2002; Park *et al.,* 2013), likely by activating the hypothalamic-pituitary-adrenal (HPA) axis and increasing circulating glucocorticoids, which at chronic levels can promote metabolic dysfunction (Nicolaides *et al.,* 2014).

In this study, the observed reduction in serum total protein concentration in the SD and RS+SD groups is also an indication of hepatic dysfunction. Proteins are essential biomolecules synthesized primarily in the liver, involved in oncotic pressure, transport, and immune function (Li *et al.,* 2021). The depletion of total protein suggests a disruption in hepatic synthetic function, increased protein catabolism, or a combination of both, as a consequence of the cellular stress associated with these challenges (Maida *et al.,* 2021). Sleep deprivation has been shown to alter the expression of genes involved in protein biosynthesis and to induce endoplasmic reticulum (ER) stress (Aboufares *et al.,* 2023). Prolonged ER stress, characterized by the accumulation of misfolded proteins, can trigger the unfolded protein response (UPR) and, if unresolved, lead to inhibition of translation and apoptosis (Botrus *et al.,* 2022). The co-exposure (RS+SD) showed a more pronounced, though not always statistically significant, decrease in albumin and globulin, pointing towards a greater combined impact on the liver's biosynthetic capacity.

One of the main features associated with physiological stress is inflammation and oxidative stress, which greatly contribute to tissue damage. The observed enzyme leakage is a downstream effect of these primary insults. Restraint stress is a well-established model for inducing oxidative stress in the liver by promoting the generation of ROS and depleting antioxidant defenses (Kumar *et al.,* 2022) Similarly, sleep deprivation has been extensively linked to increased oxidative stress in various tissues, including the liver (Santiago *et al.,* 2021). ROS can directly peroxidize lipids in the hepatocyte membrane, leading to a loss of membrane integrity and the leakage of enzymes (Zhang *et al.,* 2021). The synergistic increase in liver enzymes in the co-exposure group (RS+SD) suggests an overwhelming of the hepatic antioxidant defense system, leading to amplified oxidative damage. This is supported by studies showing that the combination of different stressors can have additive or synergistic effects on oxidative parameters (Pietrogrande *et al.,* 2021).

Furthermore, both restraint stress and sleep deprivation are potent activators of the inflammatory response. Restraint stress can increase pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 in the liver and circulation (Tan *et al.,* 2017; Aleem and Tohid, 2018). Sleep deprivation also promotes a pro-inflammatory state (Garbarino et al, 2021). These inflammatory

cytokines can directly damage hepatocytes and attract inflammatory cells, further exacerbating tissue injury and contributing to the observed rise in transaminases (Yang *et al.,* 2022). The marked elevation of ALP and GGT in the co-exposure group may also point towards stress-induced alterations in bile acid metabolism or mild cholestasis, as inflammation can disrupt bile duct function (Evangelakos *et al.,* 2021).

The reduction in serum total protein concentration observed in the SD and RS+SD groups indicates another dimension of hepatic dysfunction related to synthetic capacity. As the primary site of plasma protein synthesis, the liver's ability to produce proteins essential for oncotic pressure, transport, and immune function becomes compromised under cellular stress (Ehlting *et al.,* 2021). This depletion suggests either disrupted hepatic synthesis, increased protein catabolism, or both mechanisms operating simultaneously (Alamri, 2018). Sleep deprivation has been shown to alter expression of genes involved in protein biosynthesis and induce endoplasmic reticulum (ER) stress (Naidoo, 2012; Hakim *et al.,* 2015; Aboufares *et al.,* 2023). Prolonged ER stress, characterized by accumulation of misfolded proteins, can trigger the unfolded protein response (UPR) that may progress to translational inhibition and apoptosis if unresolved (Sisinni *et al.,* 2019).

The relative decrease in albumin and globulin fractions observed in the co-exposure group, though not always statistically significant, points toward a greater combined impact on the liver's biosynthetic capacity. The mechanistic pathway underlying these observations prominently features inflammation and oxidative stress as primary drivers of tissue damage. Restraint stress reliably induces hepatic oxidative stress by promoting reactive oxygen species (ROS) generation while depleting antioxidant defenses such as glutathione (Kim *et al.,* 2016; Chen *et al.,* 2020). Similarly, sleep deprivation has been extensively linked to increased oxidative stress in hepatic tissue (Vallianatou *et al.,* 2021: Meliante *et al.,* 2023). ROS can directly peroxidize lipids in hepatocyte membranes, compromising structural integrity and facilitating enzyme leakage (Wang *et al.,* 2023). The synergistic increase in liver enzymes in the co-exposure group suggests an overwhelming of hepatic antioxidant defense systems, leading to amplified oxidative damage. This is a phenomenon supported by studies showing that combined stressors produce additive or synergistic effects on oxidative parameters (Liess *et al.,* 2016).

Furthermore, both restraint stress and sleep deprivation independently activate inflammatory. Restraint stress increases pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6 in the liver and systemic circulation (Barron*,* 2015; Wang *et al.,* 2021), while sleep deprivation promotes a generalized pro-inflammatory state (Garbarino *et al.,* 2021). These inflammatory mediators can directly damage hepatocytes and recruit inflammatory cells, further exacerbating tissue injury and contributing to transaminase elevation (Woolbright & Jaeschke, 2018). The marked elevation of ALP and GGT in the co-exposure group may additionally indicate stress-induced alterations in bile acid metabolism or mild cholestasis, as inflammation can disrupt bile duct function.

5. CONCLUSION

In conclusion, the co-exposure of acute restraint stress and REM sleep deprivation induces a more severe hepatic injury in female Wistar rats than either stressor alone, as evidenced by the synergistic increase in liver function enzymes and the decrease in total protein. This appears to be mediated through the combined and likely amplified effects of oxidative stress, inflammatory responses, and ER stress, leading to hepatocyte damage and impaired synthetic function. These findings highlight the critical importance of managing psychological stress and ensuring adequate sleep as intertwined factors for maintaining liver health.

ETHICAL APPROVAL

This study was conducted following the guidelines of the Animal Ethical Committee of the Faculty of Basic Medical sciences, Ladoke Akintola University of Technology, Oyo, Nigeria developed guidelines for all animal studies, and these regulations were adhered to throughout the research process.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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