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Dexamethasone marginally surpasses MSC-secretome in resolving acute liver failure in mice

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EA: Methodology; Investigation; Writing — original draft, review; Data collection.

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None to declare.

Ethical approval:
The protocol of the current study and the animal use were conducted following the ethical regulations. The study protocol was approved by the ethical committee of following the ethical regulations of animal care and use of the Faculty of Science, Tanta University Animal Care and Use Committee (IACUC-SCI-TU-0184).

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The anti-inflammatory roles of Mesenchymal stem cells (MSCs) and glucocorticoids are well-reported in both preclinical and clinical studies. However, it is not clear how far MSC-secretome offers sufficient protection against acute liver failure (ALF) compared to glucocorticoids. To answer this query, acute liver failure was induced in mice by a single toxic dose (400 mg/kg) of acetaminophen (APAP). Then mice were treated with Dexamethasone or transfused with MSC-secretome, which was derived from DEX-treated bone marrow mesenchymal stem cells. The results showed that 10 nM DEX has no impact on the viability or the mesenchymal characteristics of MSCs. While the transfusion of MSC-secretome provided a significant therapeutic effect against ALF, it was marginally less effective than DEX treatment. Hepatic markers (ALT, ALP, GGT, and bilirubin) were improved more significantly in DEX-treated mice than in MSC-secretome treated group. This improvement was accompanied by marked relief in the oxidative assessed in the liver as Nrf-2, MDA, and GSH. Additionally, the normal levels of angiogenic (VEGF), and inflammatory (TNF-α) markers were effectively restored after DEX treatment. Also, both MSC-secretome and DEX resolved liver necrosis. In summary, these data suggest that dexamethasone demonstrates a better therapeutic effect than MSC-secretome in the treatment of ALF. Further studies are necessary to standardize MSC-secretome as an acellular therapeutic approach.

Key words: BM-MSCs, dexamethasone, acute liver failure, hepatoma cells, paracetamol, inflammation

Introduction

Acute liver failure (ALF) is characterized by rapid and severe liver dysfunction and may lead to death if not properly treated. Pathologically, it is associated with some features including hepatic encephalopathy and coagulopathy, which can progress to necrosis of liver tissue. Also, the pathophysiology of ALF involves a systemic inflammatory response that may lead to complications in other organs like cerebral edema and renal failure [5]. Globally, ALF occurs in humans as a result of many etiologies including drug overdose, infection with hepatitis viruses, and less frequently, autoimmune hepatitis [20]. Despite advances in critical care and liver transplantation, the mortality rate remains high, making early ALF management essential. Due to the association between liver inflammation and ALF, many authors involved the role of immunomodulatory therapies, such as corticosteroids [27] in ALT treatment. Dexamethasone, for example, showed a crucial role in managing different liver diseases due to its anti-inflammatory and immunosuppressive properties [28]. In and severe alcoholic hepatitis and autoimmune hepatitis, for instance, dexamethasone reduced liver inflammation, modulated the immune re-

sponse, prevented further liver necrosis, and improved patient outcomes. In a similar manner, treatment of liver transplant patients with dexamethasone is traditionally used to prevent acute graft rejection, contributing to patient survival. However, its use must be carefully monitored to avoid Dexamethasone-associated side effects, such as immunosuppression and subsequent inflammation. In these pathological conditions, the drug reduces the production of pro-inflammatory cytokines, limits the development of ALF-related cytokine storm, and limits further liver damage. In the past few decades, Mesenchymal stem cells (MSCs) transplantation was emerged as a promising therapeutic option for many disorders including acute liver failure (ALF). This was attributed to their regenerative potential and immunomodulatory properties [29]. These features were explained by the immunomodulation of the anti-inflammatory cytokines, released by the MSCs (secretome), which reduce inflammation and the progression of liver injury [11, 24]. Also, MSC differentiation into hepatocyte-like cells, promotion of the survival of endogenous liver cells [1], and modulation of angiogenesis and oxidative stress [7, 25] could be regarded as alternative mechanisms of MSCs-mediated ALF therapy. Additionally, the anti-fibrotic effect of MSCs was suggested due to their effect on the activity of hepatic stellate cells, which play a key role in liver fibrosis [21, 30]. The vast majority of these preclinical investigations were conducted using MSCs. Acellular regenerative approach, however, was recently adopted in which MSC-derived secretome and extracellular vesicles (EVs) replaced MSC-mediated therapy [3, 4, 14]. However, there is a lack of knowledge about the efficacy of MSC-secretome in the treatment of drug-induced liver injury (Dili) compared to Dexamethasone. To answer this query, this work was designed to compare the therapeutic efficacy of dexamethasone and MSC-secretome in the treatment of paracetamol-induced liver failure in mice.

Materials and Methods

Chemicals and reagents

Dexamethasone was purchased from *Amriya for Pharmaceutical Industries* (Alexandria, Egypt). Isolation and passaging of MSCs were carried out using cell culture media (DMEM) and supplements including L-glutamine, Fetal bovine serum (FBS), and Penicillin/streptomycin) purchased from Lonza. Liver biomarker kits including alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), and bilirubin were from *Spectrum Diagnostics* (Egypt).

Mesenchymal stem cells isolation and collection of MSC-conditioned media

Bone marrow MSCs were isolated from an adult (250 g) male Sprague-Dawley rat, following the ethical regulations of animal care and use of the Faculty of Science, Tanta University Animal Care and Use Committee (IACUC-SCI-TU-0184). Briefly, bone marrow from rat's bones (the tibia and femur) was collected in complete media containing 10% FBS and 1% penicillin/streptomycin. Cells were grown at 37°C, 95% air, and 5% CO_2. BM-MSCs conditioned media was prepared by incubating MSCs in a serum-free media for 48 h, after which media were collected, centrifuged, to remove cell debris, and utilized in mice treatment.

Annexin V/PI staining

The apoptosis assay was performed using an *Annexin-V FITC* kit (*Miltenyi Biotec*, CA, USA) following the manufacturer's guidelines. Briefly, cells were seeded in T25 flasks. After overnight incubation, cells were treated with 10 nM DEX for 24 h after which they were collected by Trypsin/EDTA, and centrifuged at 1000 rpm for 5 min. The cell pellet was resuspended in PBS and incubated with 0.25 µg/ml Annexin V in 1X binding buffer for 15 min, followed by two washes with a Wash Buffer. Cells were resuspended again in a binding buffer PI and then subjected to flow cytometry analysis.

Induction of acute liver failure in mice and grouping

Male C57BL/6 mice (25–31 g) were purchased from The National Cancer Institute, Cairo University. Animals had free access to food and water and were housed in a 12 h light/dark cycle in standard conditions. All animal experimentation was in compliance with the guide for the care and use of laboratory animals, where the experimental design was approved by the Ethics Committee of the Faculty of Science, Tanta University (IACUC-SCI-TU-0184). Mice (n=24) were randomly assigned to 4 groups, six mice each (fig. 1). Group I was left untreated as a negative control group, whereas mice in groups II, III, and IV received a single intraperitoneal dose (400 mg/kg body weight) of APAP to develop ALF. Group II mice were slaughtered 24 h after acetaminophen (APAP) injection, whereas groups III and IV were treated once with 2 mg/kg DEX via i.p injection or transfused consecutively twice with 200 µl prefiltered BM-MSC-secretome via tail vein. Animals were slaughtered one week after treatment and both blood and liver tissues were collected for further investigations.

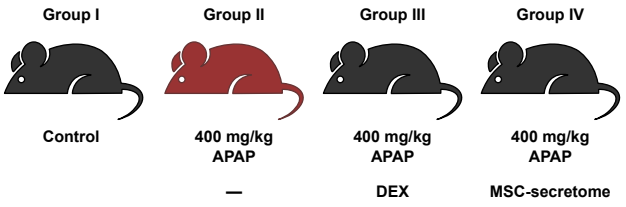

Fig. 1. Excremental design, animal grouping, and treatment protocols. Mice were divided into 4 groups. Group I (GpI) included healthy control mice. Mice in groups II, III, and IV were injected with a toxic dose of APAP to develop acute liver failure. Groups III and IV were treated with a single dose of DEX or two infusions with MSC-secretome one week apart. Mice were slaughtered one week after the last treatment

Assessment of liver function

Serum levels of ALT, ALP, GGT, and Bilirubin were measured using marker-specific kits, following the manufacturer's guidelines. In parallel, liver tissue samples were homogenized in ice-cold phosphate buffer, pH 7.2, containing 1 mM EDTA-Na2. The homogenate was centrifuged at 15,000 g at 4°C for 15 min and its protein concentration was determined in the supernatant by the Bradford assay.

Quantification of inflammatory, angiogenic, and oxidative stress markers in liver

Tumor necrosis factor alpha (TNF-α), vascular endothelial growth factor (VEGF), Nuclear factor-erythroid p45-related factor 2 (Nrf-2), and GSH were determined by ELISA kits, following the manufacturer protocols. Malondialdehyde (MDA) concentrations in liver homogenate were measured according to [19]. In this assay, 100 µl of liver homogenate sample was mixed with 300 µl perchloric acid (PCA) (0.1125 N) and thiobarbituric acid (TBA) (40 mM, 300 µl), and then placed in a boiling water bath for 60 min. After cooling methanol (600 µl) and 20% TCA (200 µl) were added and mixed for 10 s. The samples were centrifuged at 10,000 rpm for 6 min, and the MDA concentration was quantified using a standard curve, which was obtained from hydrolyzed 1,1,3,3-tetramethoxypropane (TEP) dissolved in water in different concentrations.

Histopathological analysis

For histological assessments, portions of livers were excised, and fixed in 10% formalin. After dehydration and clearance, tissues were embedded in paraffin, sectioned in 5 µm thickness, stained with hematoxylin-eosin (H&E) following the standard protocol, and examined under a light microscope.

Statistical analysis and software

Statistical analysis was performed using *Graphpad Prism 5.0* software (*GraphPad Software* Inc., San Diego, CA, USA). Data were expressed as mean ± standard deviation (SD). Mean values were compared using ANOVA test followed by Tukey test, where $P<0.05$ was considered statistically significant.

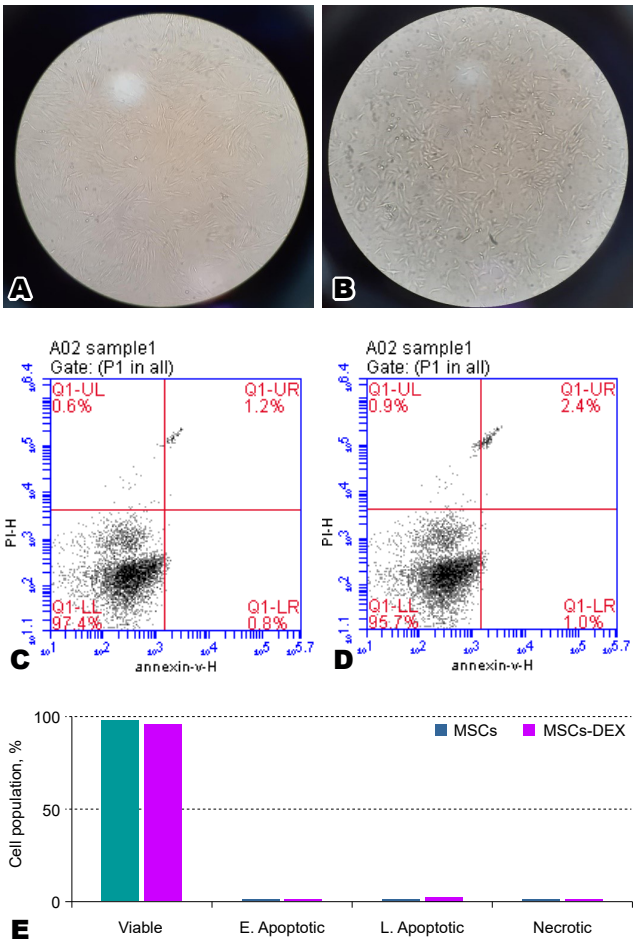

Fig. 2. Bone marrow MSCs isolation and viability assessments. MSCs were isolated from the rat's bones (tibia and fibula) (A&B), maintained to the 3rd passage, and left untreated or treated with 10 nM DEX for 24 h. Annexin V/PI dual staining was utilized to access cell viability (C&D). Bar graph "E" demonstrates that DEX-treated MSCs maintained their viability similar to the untreated cells

Results

Initially, we isolated BM-MSCs from rat's bones. Adherent cells, maintained in standard culturing conditions, demonstrated a typical spindle, fibroblast-like shape (fig. 2). Incubation of cells in a complete media containing 10 nM DEX for 24 h did not affect cell morphology and viability. To ensure that, cells were dually stained with Annexin-V and PI, where both untreated and DEX-treated cells did not show significant apoptosis nor necrosis (fig. 2).

In parallel, we developed ALF in mice through the administration of a single toxic dose of APAP (400 mg/kg body weight). Signs of ALF were authenticated by monitoring the serum levels of liver function markers including ALT, ALP, GGT, and bilirubin, where all markers were significantly increased compared to the healthy group ($P<0.001$ with all serum markers) (fig. 3).

Cells were utilized to prepare MSC-secretome. Next, 24 h after the development of ALF, mice were treated with a single dose of 2 mg/ml DEX (in group III) or transfused twice with MSC-secretome. Mice treatments led to a significant decrease in the serum ALT, ALP, GGT, and bilirubin (fig. 3). The improvement in these markers was more pronounced in DEX-treated mice compared to the ALT and ALP. Since APAP development is associated with a significant increase in oxidative stress, we measured three oxidative stress markers. Treatments with both MSC-secretome and DEX were associated with the amelioration of the oxidative stress markers (Nrf-2, MDA, and GSH) in the liver (fig. 4).

Additionally, the high levels of the inflammatory marker (TNF-α) observed in the liver of ALF mice, significantly decreased in treated mice particular in DEX-treated mice.

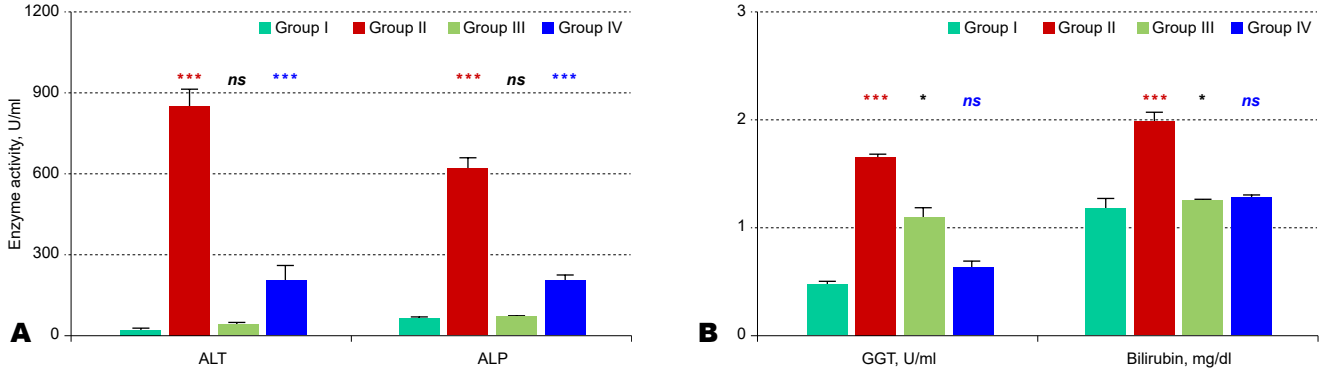

Fig. 3. Effect of treatment of ALF mice with DEX and MSC-secretome on serum liver markers. Mice treatment with DEX or MSC-secretome ameliorated ALT, ALP, GGT, and bilirubin. Significant changes, compared to healthy mice (GpI), are indicated by (*), where (*) or (***) refer to P<0.05 or P<0.001, respectively; (♦) refers to a significant difference between the indicated groups versus the DEX-treated group (GpIII). Abbreviations: ALF — acute liver failure, ALT — alanine aminotransferase, ALP — Alkaline phosphatase, GGT — gamma-glutamyl transferase, and Bili — total bilirubin

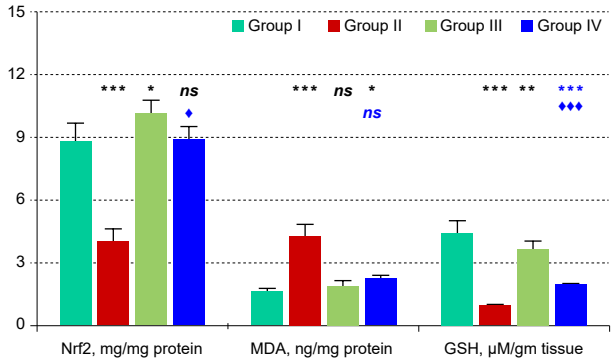

Fig. 4. Oxidative stress markers in the liver. Mice in groups III and IV were treated with DEX or MSC-secretome, respectively.
The results of Nfr-2, MDA, and GSH are shown as means (± SD) (n=6) for each group (n=6). Mice in groups II, III, and IV were injected with APAP to develop ALF. Statistical significances between treated groups are indicated as (*) or (♦) to compare the indicated groups versus control or DEX-treated groups, respectively.
Nrf-2: MDA: Malondialdehyde, GSH reduced glutathione

A similar improvement pattern was observed in the angiogenic marker (VEGF) (table 1). Furthermore, these biochemical changes were accompanied by significant resolving of the necrotic and inflammatory changes in the liver tissue of ALF mice (table 1) one week following treatments.

Table 1. Mean values of TNF-α and VEGF in liver

Groups	Group I (Control)	Group II (ALF)	Group III (DEX)	Group IV Secretome
TNF-α	24.66±2.51	87.66±4.5 ***	21.33±2.51 ns	46.33±5.1 ***,♦♦♦
VEGF	86.66±4.04	147.33±5.5 **	71±6.55 *	100±9.16 ***,♦♦♦
Histological score	0.0	0.5	0.0	0.578

Note. *** — P<0.00 — significant difference compared to healthy group (Gp1); ♦♦♦ — P<0.001 — significant difference compared to DEX-related group (GpIII)

Discussion

This work suggests that dexamethasone (DEX) can cure paracetamol-induced liver failure more effectively than transfusion with MSC-secretome. We found that the liver function markers were improved in mice treated with DEX more efficiently compared to both untreated ALF mice and mice transfused twice with MSC-secretome. Also, the pro-inflammatory marker (TNF-α), the angiogenic (VEGF), and the oxidative stress markers were ameliorated more efficiently in DEX-treated mice. Additionally, both treatment methods resolved hepatic necrosis. APAP-induced ALF is typically background with rapid liver cell injury and oxidative stress. In agreement with previous studies [13], ALF pathogenesis was associated with the development of significant hepatic inflammation (assessed by TNF-α) and enhanced production of VEGF. The healing effect of MSCs against acute and chronic liver failure was repeatedly reported as cell-based regenerative therapy [21, 23]. Herein, we utilized acellular approach that may offer more advantages over MSC treatment. To avoid transfusion-associated inflammation, we treated MSCs, from which the secretome was collected, with 10 nM DEX for 24 h. This concentration did not induce any morphological or apoptotic changes in MSCs. Although both DEX and MSCs demonstrate anti-inflammatory effects [16, 22], they, however, adopt different mechanisms. The MSC-related anti-inflammatory role is attributed to the cytokines they release, such as IL10, and TGF-β, which modulate T cells and macrophages to reduce inflammation and inhibit the production of pro-inflammatory cytokines such as TNF-α and IL-6 [10]. Glucocorticoids, however, adopt different mechanisms as they activate glucocorticoid receptors (GR) leading to suppression of NF-kB and decreasing the production of TNF-α and IL1β [28]. Although both DEX and MSC-secretome affect the same immune cells, the former (DEX) may target a wide range of other non-immune cells as

Table 2. Factors affecting the variability in MSC-secretome composition

Modulatory factors	Secretome components	Description	References
Source	Growth and angiogenic Factors	BM-MSCs, AT-MSC, and, and UC-MSCs may secrete different levels of growth factors.	[6]
	Cytokines	The cytokine profile differ based on tissue origin.	[12]
	EVs (e.g., exosomes, microvesicles)	EVs-content vary depending on MSC source.	[26]
Culture Conditions and microenvironment	Hypoxic condition	Increase the secretion of angiogenic factors.	[2]
	Media and supplements	Serum-free conditions may alter the secretion of immunomodulatory cytokines.	[15]
	Cell passaging	Increased passaging may lead to reduced secretion of certain factors.	[8]
	Mechanical Stress	Mechanical stimulation may increase the secretion of MMPs.	[21]
	Preconditioning	Chemical preconditioning may modify the MSC secretome towards a specific lineage.	[22]

well. Beside its potent effect, DEX has the privilege as FDA-approved drug, where it is widely prescribed for inflammatory, rheumatoid arthritis, and autoimmune diseases [16]. As the liver is the main xenobiotic metabolizing organ, DEX is mainly converted via CYP3A4 and other CYPs into 6β-hydroxy-dexamethasone (6βOH-DEXA) and 6α-hydroxydexamethaaone (6αOH-DEXA) [9]. Accordingly, it is anticipated that the therapeutic effect is implemented by DEX and its metabolites as well. Also, its overdose-related side complications and its involvement in glucose metabolism are well-identified. Although MSC-secretome presented a significant protection against ALF, it is still in the preclinical phase, and its promising therapeutic role is challenged by the relatively complex and costly preparations, unstandardized production, less-optimized delivery protocol, and ethical concerns.

Furthermore, it demonstrates significant variability in the bioactive molecules they include according to the source tissue of MSCs, the culture conditions, stability, and other aspects (table 2). More importantly, MSC-secretome therapy has some concerns about enhancing the proliferation and growth of endogenous cancer cells [26]. These concerns make it difficult to predict their therapeutic outcomes in different diseases.

Conclusively, this work suggests that treatment of ALF with DEX or MSC-secretome resolved hepatic necrosis and improved the liver function. However, DEX demonstrated better outcomes as indicated by the effective amelioration in liver function, inflammatory, and angiogenic markers in addition to the restoration of hepatic architecture. Although MSC-secretome demonstrated good healing effects, it is challenged by many obstacles that are required to be addressed through further research. Standardization of preparation and delivery protocols is essential to ensure their safe and effective application.

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Дексаметазон незначно перевершує МСК-секретом у вирішенні гострої печінкової недостатності у мишей

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Протизапальну роль мезенхімальних стовбурових клітин (МСК) і глюкокортикоїдів добре описано як у доклінічних, так і в клінічних дослідженнях. Однак неясно, наскільки МСК-секретом забезпечує достатній захист від гострої печінкової недостатності (ГПН) порівняно з глюкокортикоїдами. Для вивчення цього питання у мишей спровокували гостру печінкову недостатність одноразовою токсичною дозою (400 мг/кг) ацетамінофену (APAP). Потім мишей лікували дексаметазоном або переливали MSC-секретом, отриманий з оброблених DEX мезенхімальних стовбурових клітин кісткового мозку. Результати показали, що 10 нМ дексаметазону не впливають на життєздатність або мезенхімальні характеристики МСК. Хоча переливання МСК-секретома забезпечувало значний терапевтичний ефект проти ГПН, воно було дещо менш ефективним, ніж лікування дексаметазоном. Печінкові маркери (АлАТ, ЛФ, ГГТ і білірубін) вираженіше покращилися у мишей, які отримували дексаметазон, ніж у групі, яка отримувала МСК-секретом. Це покращення супроводжувалося помітним полегшенням окислення, оціненого в печінці як Nrf-2, MDA та GSH. Крім того, нормальні рівні ангіогенних (VEGF) і запальних (TNF-α) маркерів були ефективно відновлені після лікування дексаметазоном. Крім того, як МСК-секретом, так і дексаметазон усувають некроз печінки. Ці дані свідчать про те, що дексаметазон демонструє кращий терапевтичний ефект, ніж МСК-секретом, у лікуванні ГПН. Необхідні подальші дослідження для стандартизації МСК-секретома як безклітинного терапевтичного підходу.

Ключові слова: КМ-МСК, дексаметазон, гостра печінкова недостатність, клітини гепатоми, парацетамол, запалення

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