The expression levels of genes involved in JNK signaling decrease by aging in the liver

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Abstract

Aim: The c-Jun NH2-terminal kinases (JNK) signaling pathway is an important signaling pathway in liver regeneration. it was planned to investigate the expression levels of Mitogen Activated Protein Kinase Kinase (MKK)-4 and -7 (MKK7), which are mediator molecules involved in the JNK signaling pathway and Activating Transcription Factor (ATF) 2 transcription factor genes, which are in the last stage of the signaling pathway, in liver tissues in young and old mice. In addition, it was aimed to examine the ultrastructural changes caused by aging in hepatocytes.

Material and Methods: We examined *MKK4, MKK7* and *ATF2* expression levels by using Real Time Polymerase Chain Reaction (RT-qPCR) method. Transmission electron microscopy (TEM) was used to observe the ultrastructural changes of hepatocytes.

Results: While *MKK4* and *ATF2* gene expressions reduced in the liver of aged mice, *MKK7* gene expression did not change. In TEM examinations, granular endoplasmic reticulum loss and mitochondrial damage were observed in elderly individuals.

Conclusion: According to these results, spontaneous liver damage that can be seen in aged subjects may be caused by disruption in cellular signaling pathways and organelle damage in hepatocytes.

Introduction

In recent years, developments in the field of medicine have extended life expectancy and the number of elderly individuals has been increasing gradually. Aging is characterized by disruption of metabolic pathways and cellular aging. The formation of senescent cells is accompanied by telomere shortening, DNA damage, epigenetic changes, oxidative stress and mitochondrial dysfunctions. Weakening of the immune system in the aging process, decrease in the power of the antioxidant system, and decrease in the regeneration capacity of the liver cause many diseases [1]. The liver is also an organ affected by aging. Although there is no liver disease specific to advanced age, it is known that aging causes changes in hepatic morphology and functions [2]. Aging causes the clinical course of liver diseases to be different from that of young people. The higher incidence of diseases such as hepatitis B and C in individuals over 60 years of age, the increased incidence of HCC (hepatocellular carcinoma) , the increase in the incidence of autoimmune hepatitis, especially in menopausal women, and the incidence of NASH (non-alcoholic steatohepatitis) make aging an important phenomenon in terms of liver diseases [2, 3].

Today, the effective treatment of end-stage liver disease is liver transplantation, but liver transplantation is rarely performed in advanced ages and the success of liver transplantation in individuals over 60 years of age is relatively low compared to young people. One of the reasons for transplant failure in advanced ages is the decrease in the regeneration capacity of the liver. Decreased regeneration capacity poses a risk for both the donor and the recipient [4, 5].

It is thought that the JNK signaling pathway may play a role in the molecular mechanism of liver regeneration [6]. The JNK, a member of the MAP kinase (MAPK) family, is a regulatory protein involved in the transmission of various signals received from outside the cell into the cell. The

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JNK signaling pathway takes role in regulatory events, including apoptosis, growth control and transformation [7, 8]. It has various isoforms such as JNK1, JNK2 and JNK3, depending on the substrates they target. It is known that the isoforms JNK1 and JNK2 are expressed in all tissues. However, JNK3 is expressed only in testis, brain and heart [9, 10].

Stimuli such as cytokines, growth and stress activate small GTPases, then signals are transmitted to MEK kinase and MEK homologues (SEK1/MKK4/JNKK) and finally JNK signaling pathways are activated. That also activates several transcription factors such as ATF2, Elk-1, p53, c-Jun and non-transcription factors B-cell lymphoma 2 (Bcl-2) family [7]. MAPK kinase-kinase (MEKK) protein is known to be activated in various cellular stress situations. After activation of MEKK, it activates MKK4 and/or MKK7, which in turn activates JNKs [11].

Since ageing is considered as a dysregulation of most of the physiological systems together with various regulating mechanisms in a cell; it also affects the JNK signaling pathway. During ageing, cells lose their capacity to proliferate, differentiation and respond to environmental factors [12]. In case of liver regeneration, DNA synthesis delayed and reduced significantly after partial hepatectomy in aged rats compared to young ones. Ishigami et al. discovered that stimulation of primary hepatocytes with epidermal growth factor does not elevates the rate of DNA synthesis in aged rats in comparison to young animals [13]. Besides that, ageing affects different molecular events and signaling pathways in a cell. Atadja et al. demonstrated that regulation of gene expressions are altered by aging since hyperphosphorylation of serum response factor and serum response element binding activity significantly reduced in human diploid cells [14]. Riabovol et al. reported that the Activator Protein 1 (AP-1) decreases in human fibroblasts during the aging process, and therefore, the proliferation response of senescent cells to mitogenic stimuli will decrease [15].

In this study, we investigated gene expression levels of two kinase proteins MKK4, MKK7, and also transcription factor, ATF2, which involves in JNK signaling pathway cascade in liver homogenates of old and young mice. Thus, we aimed to reveal the effect of aging on the expression of these genes. In addition, ultrastructural analysis of liver tissues was performed.

Materials and Methods

Animals and Experimental Groups

Male Balb/C mice purchased (12 week and 18 months) from Inonu University experimental animal production and research center were used (Malatya, Turkey). Experimental procedures applied to the animals were carried out in accordance with the ethical rules determined by the Inonu University experimental animal ethics committee (Ethics committee number 2018/A-11). The mice were randomly grouped into two with ten mice in each within their age groups. Power analysis was performed to determine the number of animals that should be included in the groups. In the power analysis, it was calculated that there should be at least 10 subjects in each group.

Table 1. Properties of primers used in RT-qPCR.

Gene	Band Size	RefSeg Number	Reference Position
MKK4	89bp	NM_009157	1019
MKK7	93 bp	NM_001042557	587
ATF2	92 bp	NM_001025093	3633
TGF-β	63 bp	NM_011577	1526
GAPDH	140 bp	NM_008084	478

Mice were sacrificed under high-dose anesthesia to obtain liver and blood tissues. Afterwards, gene expression and ultrastructural analyzes were performed.

RT-qPCR Analysis

The quantitative real-time PCR method was used to measure the mRNA expression level of MKK4, MKK7, ATF2, Transforming Growth Factor Beta (TGF-β) as a marker gene to detect fibrosis in the liver and housekeeping gene Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) genes. Liver from the groups was cut into small pieces for RNA purification and stored in a -80 ºC freezer until analysis. RNA purification was performed by using the RNeasy Plus Mini Kit (Qiagen, Germany Cat. No. 74134). Then, after confirming the purity of the RNA samples obtained by measuring wavelengths of 260/280 nm, complementary DNA (cDNA) synthesis was started. RT2 HT First Strand Kit (Qiagen, Germany Cat.No.330411) was used to obtain cDNA. The obtained cDNA samples were stored at -20 ºC until RT-qPCR was performed. Real-time PCR was performed in Rotor Gene Q (Qiagen, Germany) by using RT^2 SYBR Green qPCR Mastermix (Qiagen, Germany Cat. No. 330501) and RT2 qPCR Primer Assay (Qiagen Germany) with the primers listed in Table 1.

A separate mix was prepared for each gene. The procedure is briefly as follows. For each sample, 1 μl of primer mix, 1 μl of cDNA, 12.5 μl of Syber Green mix and 10.5 μl of PCR grade water were added and mixed. PCR conditions were carried out as recommended by the company (Figure 1A).

The obtained PCR products were subjected to agarose gel electrophoresis. Confirmed obtained from targeted gene regions (Figure 1B)

$2^{-\Delta\Delta Ct}$ method was used to determine the change in MKK4, MKK7, ATF2, TGF-β gene expressions between groups.

Microscopic Evaluations

In order to determine the histological damage and its level, liver sections were fixed in 10% neutral buffered formalin and sections of tissues were sliced in 5 μm size by using a microtome (Leica RM2145) from paraffin blocks. Following that, samples were stained with Mayer's hematoxylin and eosin (H&E) method.

Ultrastructural Analysis

For electron microscopic examination, liver samples cut into 2 mm3 size were first fixed in 2.5% glutaralde-
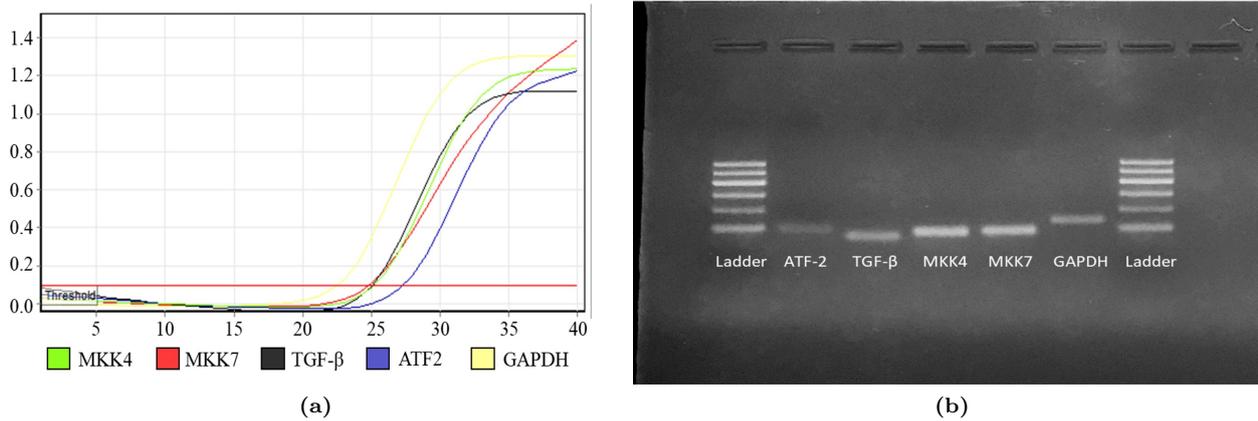
Figure 1. Amplification curves (A) and agarose gel electrophoresis (B) of the RT-qPCR results. MKK4, MKK7, ATF2, TGF-β mRNA, DNA samples after RT-qPCR and visualization of those PCR products at gel electrophoresis (at %2,5 gel electrophoresis, 1 hour, 90 Volt running conditions. The first and last lines indicate the 100bp DNA Marker (GelPilot, Qiagen), the lines in between show the ATF2 (92 bp), TGF-β (63 bp), MKK4 (89 bp), MKK7 (93 bp) and GAPDH (140 bp) gene, respectively.

hyde. Subsequently fixed (post-fixed) 1% osmium tetroxide (OsO_4) and embedded in Araldite CY 212. Then, sections (Ultra-thin- 80 nm) contrasted with uranyl acetate and lead citrate were examined by using Zeiss Libra 120 (Carl Zeiss NTS GmBH., Oberkochen, Germany) TEM (16).

Statistical Analysis

Statistical analyzes of the obtained $2^{-\Delta\Delta Ct}$ values were performed using the IBM SPSS version 25.0 statistical software package (Chicago, IL, USA). Whether the groups had a normal distribution or not was determined by the Shapiro-Wilk test. Analysis of the data not showing normal distribution was done with the test Mann-Whitney U. $p < 0.05$ was considered as statistically significant.

Results

Expression Levels of Genes

Expression levels of MKK4, MKK7, ATF2 and TGF-β genes in liver tissue of young and old mice were compared. We discovered that, ATF2 and MKK4 gene expressions decreased in old mice compared to young animals ($p < 0.05$) (Figure 2) (Table 2). We did not observe a significant difference at MKK7 expression levels of young and old mice. Generally, TGF-β expression increased during fibrosis in the liver. We did not observe a significant elevation on TGF-β expression in old mice (Figure 2) (Table 2).

Histological Analysis

In the histological evaluations we made on the liver tissue, normal histological appearance was observed in the H&E stained young liver sections (Figure 3a).

In the old group, cytoplasm showing hydropic changes in hepatocytes, pycnotic nuclei and minimal inflammatory cell infiltration were observed (Figure 3b).
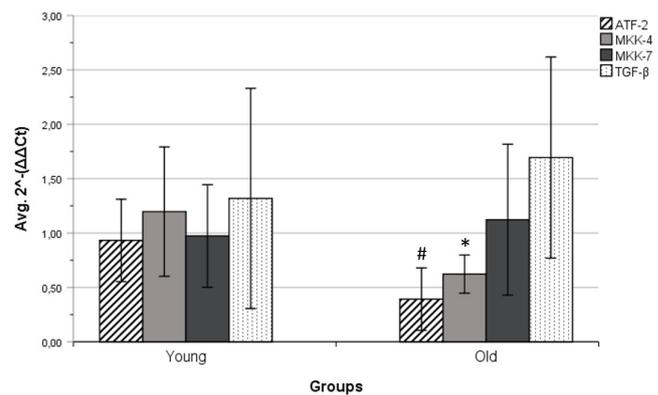
Figure 2. The data are presented mean $\pm$ SD $2^{Avg-\Delta(\Delta Ct)}$ values. * and # indicate significant decreases of MKK4 and ATF2 between young and old groups, respectively.

Table 2. Exchanges between groups

Gene	Young Grup Average $2^{-\Delta\Delta Ct}$	Old Grup Average $2^{-\Delta\Delta Ct}$	p Value
ATF2	0.93	0.39	p = 0.012
MKK4	1.22	0.62	p = 0.21
MKK7	0.97	1.12	p = 0.958
TGF-β	1.32	1.69	P = 0.172

(a)   **(b)**

(c)   **(d)**
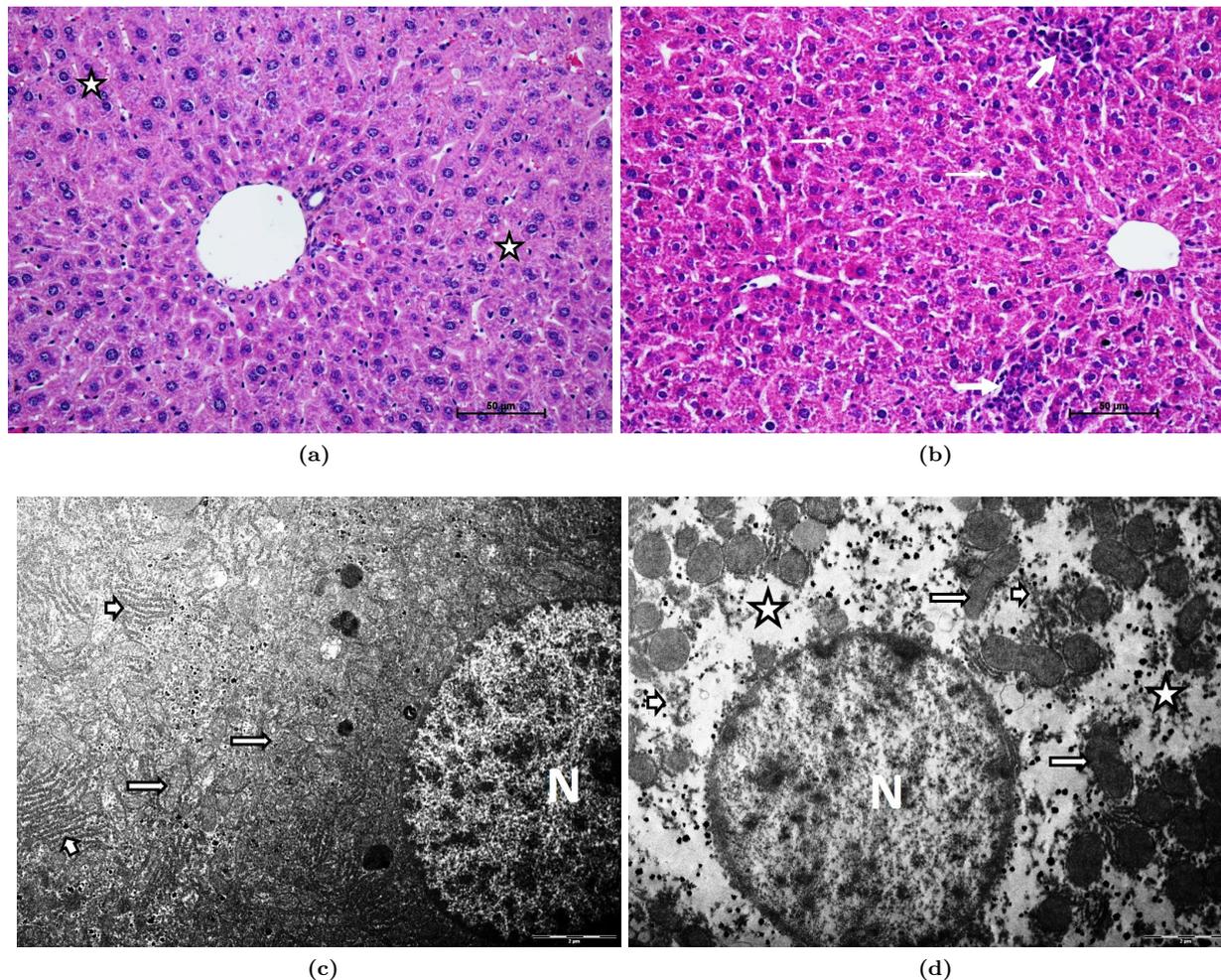
Figure 3. H&E staining images of sections obtained from liver tissues (a-b); Liver parenchyma and hepatocytes (star) are shown in normal histological appearance in the young group (a, x20); Minimal inflammatory cell infiltration (thick arrow) and hydrophic degeneration of hepatocytes (thin arrow) were observed in the old group (b, x20). Ultrastructural images of sections obtained from liver tissues (c-d); Normal ultrastructural structure was observed in the young group hepatocyte cell nucleus (N), mitochondria (long arrow), granular endoplasmic reticulum (short arrow) (c, TEMx6300); cell nucleus (N), intracellular edema (star), endoplasmic reticulum damage (short arrow), mitochondrial matrix condensation and elongated mitochondria (long arrow) were observed in the old group (d, TEMx6300).

In electron microscopic examinations, liver sections of the young group were in normal ultrastructural structure (Figure 3c).

The ultrastructural changes observed in the aged liver tissue were as follows; Intracellular edema in hepatocytes, damage and reduction in the granular endoplasmic reticulum and condensation of the mitochondrial matrix (Figure 3d).

Discussion

The loss of function in organs due to aging causes people to lose their lives [6]. The liver also gradually loses its functionality due to aging, and this situation is known to increase the risk for liver diseases [17]. It is known that the MAP kinase pathway plays an active role in the dysfunction of cells and tissues is associated with diseases with increased incidence by aging such as, cancer, Parkinson and Alzheimer diseases [18]. Our aim in this study was to

measure the gene expression levels of MKK4 and MKK7 in the JNK signaling pathway, as well as ATF2, one of the target molecules of regeneration-related signaling, and TGF-β, a liver injury marker, in the liver of young and old mice. We also demonstrated the differences of histological views of liver tissues by performing H&E staining and ultrastructural examinations in young and old mice. As a result, we found that MKK4 and ATF2 gene expressions were decreased in elderly individuals compared to young mice. In addition, we revealed the aging-related changes in hepatocytes with microscopic examinations.

JNK and p38 sub-pathways, members of the MAPK family that are involved in the regulation of many cellular functions, are activated in various cellular stress situations such as hypoxia or oxidative stress, as well as the stimulation of proinflammatory cytokines such as TNF and IL-1β. The JNK pathway is activated by activation of specific MAP2Ks (MKK4 and MKK7) [19, 20]. While MKK7

is a specific activator of JNKs, MKK4 can phosphorylate both JNK and p38 MAPKs [21]. It is known that JNK signaling in hepatocytes plays an active role in cell death and survival, tumorigenesis, differentiation and proliferation. It also plays a role in fibrosis and inflammation in non-parenchymal liver cells such as HSC and Kupffer cells [22].

The liver has an extremely high amount and density of mitochondria compared to other organs. Hepatocyte mitochondria provide the energy needed to regulate ammonia detoxification and anabolic pathways. Mitochondria are the main actors in maintaining the balance between cell survival and cell death, especially in hepatocytes, where they trigger the intrinsic pathway of apoptosis and also take part in necrotic cell death [23]. The incidence of liver diseases increases with aging in humans, and mitochondrial dysfunctions have been identified in liver tissues of them [24]. Maria et al. stated that mitochondrial dysfunction is common in liver samples taken from patients during transplantation and that improving mitochondrial function will make an important contribution to the treatment of different liver diseases [25]. In our study, we observed that mitochondrial matrix condensed in the hepatocytes, the mitochondria are elongated and bent, and the number of mitochondria decreased in TEM examinations.

Many chronic liver diseases are known to be associated with oxidative stress. Mitochondrial dysfunction in aged tissues is an important source of reactive oxygen species (ROS) which creates oxidative stress. Increased ROS production has been described in most liver pathologies. This creates oxidative stress. In addition, the liver is exposed to high amounts of oxidative stress due to its high metabolic activity [26]. Also, it is known that weakened antioxidant system and the elevated mitochondrial dysfunctions induce apoptosis in elderly individuals [27-30]. The JNK pathway is frequently activated by the effect of oxidative stress accumulating in the liver, and that often results in apoptosis. In our study, despite the decrease in gene expression of MKK4, the expression of the other protein carrying the signal required for apoptosis, MKK7, did not change. We believe that is probably because elderly individuals use MKK7 more in the pathways leading to apoptosis in the liver. In addition, it is known that the selection between MKK7 and MKK4 is more dependent on the type of stimulus, and proinflammatory cytokines such as IL-1 and TNF are mostly associated with MKK7 [31].

Since JNK pathway plays a role in proliferation and regeneration of liver tissue, it becomes more important in the aging process. Wuestefeld [1] et al. studies identified the MKK4, MKK7 and ATF2 genes as key genes in liver cell regeneration. They said that the suppression of MKK4 at the molecular level with RNA interference is compensated by the increase in MKK7 phosphorylation, which increases JNK1 phosphorylation. Thus, ATF2 phosphorylation further down the signal pathway increased, which they said accelerated the entry and progression of hepatocytes into the cell cycle during liver regeneration. Similarly, Rmilah et al. [32] reported that, suppressing MKK4 benefits post-hepatectomy regeneration in pigs. The decrease in MKK4 gene expression in our elderly individuals may be an effective response to addressing the need for regeneration,

which is greater than in younger individuals. However, it is known that the regeneration capacity is even lower in older individuals than in younger individuals [4], As a matter of fact, Wuestefeld et al. [1] observed in the same study that MKK4 deficiency is compensated by MKK7 and therefore JNK1 and ATF2 phosphorylation increases and regeneration increases, but in contrast, in our study, ATF2 gene expression decreased in the elderly and the target molecule to be phosphorylized with old age decreased. In many liver diseases, TGF-β's mRNA and protein levels have been shown to increase slightly in older mice, but it was not statistically significant, perhaps it would have made sense if there had been a study involving more animals.

It has been reported that the volume and regeneration capacity of the liver decrease with aging [1]. Although this phenomenon has long been reported, the molecular reason for the loss of regeneration in the liver due to aging has not yet been fully elucidated [33, 34]. This can be explained by the rough endoplasmic reticulum defect. As we have determined in our electron microscopic examinations, there has been a reduction on organelle density by aging. In particular, the rough endoplasmic reticulum and thus ribosomes appear to be destroyed, which may result in loss of protein production and regeneration function. In addition, it is observed that active euchromatin regions in the hepatocyte nucleus of aged mice are much more than in young mice, this can be considered as a general increase in gene expression for regeneration in old age, but protein synthesis may not occur due to the absence of ribosomes. In addition, the cause of edema observed in the sections may be due to damage to the cell and organelle membranes.

As known, in some cases changes in the amount of mRNA are not reflected in protein levels due to post transcriptional mechanisms, for this reason, it is appropriate to measure the level of the relevant proteins of MAPK pathway in liver homogenates in the future, furthermore, the investigation of the MKK3-MKK6/p38 pathway, another branch of the signal pathway, may provide guidance for full clarification of the mechanism. In addition, the lack of an animal group that corresponds to the middle age group in the study is one of the most noticeable shortcomings of the research.

Conclusion

Considering the results of the study, aging causes damage to the liver mitochondria, as in many liver diseases, and this situation necessitates the aging and mitochondrial damage in the liver to be considered together. There is still no more effective method than liver transplantation in the treatment of end-stage liver disease but decreased regenerative capacity in older individuals is one of the limiting factors in transplant therapy, so illuminating the mechanisms affecting regeneration in older individuals will give us therapeutic strategies to increase regenerative capacity. In this context, it is important for liver health in elderly individuals to have targets to increase regenerative capacity and to eliminate numerical and functional mitochondrial losses.

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