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Effects of Human Amniotic Fluid on Fracture Healing in Rat Tibia

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Background. **Human amniotic fluid (HAF), including hyaluronic acid (HA) and several growth factors, has been used experimentally in tendon, nerve, and cartilage regeneration and in bone defects because of its positive stimulating effects on regeneration potential. This study was performed to investigate whether HAF was effective on fracture healing.**

Materials and methods. **We created 36 tibial fractures in 20-week-old Wistar rats that were divided into three groups. In group 1, fracture lines were instilled with HAF collected at 18th week of the gestation and, in group 2, fracture lines were instilled with HAF obtained at the end of the gestation. HAF which was collected from different period of gestation was used, because the concentration of HA and growth factors in HAF varies considerably during gestation. Group 3 was used as an operative control group.**

Results. **Fracture-healing score was highest in group 1 radiologically at the 3rd and 5th week ($P = 0.037$, $P = 0.018$, respectively). In the scintigraphic evaluation, metabolic activity at the fracture site was observed in group 1 more than the others at the 3rd week ($P = 0.010$). Histologically, the highest scores were obtained from group 1 as compared to other groups at the 3rd and 5th week. In the 5th week, predominant cartilage with some woven bone was observed in group 3, while predominantly woven bone with some cartilage was observed in group 1 ($P = 0.039$).**

Conclusions. **Our data suggest that HAF had a positive effect on fracture healing in rat tibia, and also this positive effect was observed more in group 1.**

Key Words: **human amniotic fluid; growth factor; hyaluronic acid; fracture healing; rat; fibroblast growth factor.**

INTRODUCTION

Fracture healing is a specialized type of wound-healing response which ultimately reconstructs the integrity of bone and its biomechanical properties. Fracture healing is generally free of problems, but sometimes impaired healing may occur due to different problems. For many years researchers have been enhancing alternative treatments to impaired fracture healing [1, 2]. Mechanical stimulation, electrical stimulation, ultrasound stimulation, osteogenic, osteoconductive, and osteoinductive methods, and growth-promoting proteins were suggested to increase the fracture healing as biophysical and biological methods.

Several growth-promoting factors have been identified after the fractures, such as transforming growth factor-beta (TGF-β), fibroblast growth factor (FGF), platelet-derived growth factor, interleukin-1, and interleukin-6 [1, 2]. One of these factors is FGF, which has angiogenic properties and mitogenic activity on the osteoblast lineage [2–4].

The rich contents of growth and trophic factors such as epidermal growth factor (EGF), FGF, insulin-like growth factors I and II (IGF-I and IGF-II) that are critical for development was identified in human amniotic fluid (HAF) [3, 5–7]. Additionally, hyaluronic acid (HA), hyaluronic acid stimulating activator (HASA), chondroitin 4- and 6-sulfate, dermatan sulfate, and heparan sulfate have been identified in HAF [8].

HA is a high molecular weight linear polysaccharide found in most tissues, especially connective tissues and body fluids. It has been assigned an important role in embryonic development [9]. HA is capable of accelerating new bone formation through increased mesenchymal cell differentiation in bone wounds [10, 11].

It has been reported that HAF has a changing composition of both growth factors and HA throughout gestation [6–8, 12–15]. HA concentration in HAF is 20 times higher mid-gestation (16- to 20-weeks' gestation) than near term [8]. On the other hand, amniotic fluid EGF concentrations correlate with gestational age and it increases toward term [15].

We hypothesized that HAF including both growth factors and HA would have a positive stimulating effect on fracture healing. So we aimed to investigate the effects of HAF on fracture healing in a rat tibia fracture model. Additionally, it was also evaluated whether the changing composition of HAF effects fracture healing or not. Therefore HAF, which was collected both at 18th week of the gestation and at end of the gestation, was used.

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MATERIALS AND METHODS

In this study, conducted in an experimental research laboratory, the right tibias of 36 male Wistar rats, which had standardized fracture configuration, were used following approval by the ethical committee of the university and local authorities. The animals, with a mean age of 20 weeks (range, 19 to 21 weeks) and a mean body weight of 296 g (range, 284 to 322 g), were randomly and equally divided into three groups. Group 1 was treated with 0.3 mL HAF (collected at 18th week of the gestation) instilled into the fracture lines. Group 2 was treated with 0.3 mL HAF (obtained at end of the gestation) instilled into the fracture lines. Nothing was instilled into the fracture lines in Group 3, used as an operative control group because our aim was the comparison between the experimental groups and the normal fracture healing. Six rats from each group were euthanized at the 3rd week and the remaining six were euthanized at the 5th week. Radiological, scintigraphic, and histological examinations were made on the right tibias to evaluate the fracture healing.

The HAF, which was used in group 1, was obtained from a normal pregnancy by diagnostic amniocentesis, performed at the 18th week of gestation under sterile conditions, centrifuged at high speeds, with the serum collected, and stored at −20°C until use. The HAF used in Group 2 was collected at the time of membrane rupture from a different pregnancy at the end of the gestation. Then its serum was stored using the same protocol.

Surgical Technique

After the adaptation period of 7 days, the rats were not fed 4 h before surgery. Fifty milligrams per kilogram of ketamine hydrochloride (Ketalar; Pfizer, Istanbul, Turkey) and 10 mg/kg of xylazine hydrochloride (Rompun; Bayer Healthcare, Leverkusen, Germany) were injected intramuscularly as anesthetic. If necessary, a further 15 mg/kg dose of ketamine hydrochloride was administered intraoperatively. The rats were placed in the supine position and skin overlying the right tibia was swabbed before and after surgery with a 10% povidone iodine solution (Batticon; Adeka, Samsun, Turkey). The right tibia was exposed through a longitudinal skin incision performed at the front edge of the tibia. Standardized closed tibia and fibula fractures were inflicted manually on the middle of the bone as previously described by An *et al.* [16]. Standardization of fracture configuration was determined radiographically. Any rats with comminuted fractures were excluded from the study. Tibia fractures were fixed with intramedullar fixation using 21-G sterile needles. A single 0.3 mL of HAF injection was instilled directly into the fracture lines of each rat in Groups 1 and 2. The skin incision was closed with interrupted sutures using nonabsorbable polypropylene sutures (Trefilen, Degsan, Trabzon, Turkey).

The animals were permitted full weight-bearing and unrestricted movement upon awakening from anesthesia. They were kept separately in cages, allowed free cage activity, and fed *ad libitum* with commercial laboratory animal fodder. The light/dark cycles were 12 h/12 h. All surgeries were performed by the same surgeon to guarantee the complete homogeneity of the results.

At the end of the experiment, the animals were anesthetized with ketamine and xylazine, and then scintigraphic procedures were done. Later the animals were euthanized by cervical dislocation. The tibias were harvested, stripped of the muscle tissues, X-rayed, and stored in 10% formaldehyde for histological procedures.

Radiological Evaluation

Radiographs were taken for evaluation at the time of euthanasia, and the fracture healing of each specimen was graded for callus maturity by an independent observer who was blinded to the identity of the specimens using the Lane–Sandhu Scoring System [17] as follows: 0 = no healing; 1 = callus formation; 2 = starting ossification; 3 = starting to lose fracture line; 4 = complete knitting of bone. The mean radiographic score was calculated for each treatment group.

Scintigraphic Evaluation

All studies were performed using a large field-of-view gamma camera (Siemens E-cam Dual Head) equipped with a low-energy, parallel hole, high-resolution collimator, and Tc99m methylene diphosphonate was used as the tracer. A dose of 74 MBq of Tc99m methylene diphosphonate was injected into a tail vein as a bolus. The rats were placed on the imaging table in the supine position and static images were obtained 3 h after injection. The following quantitative evaluation was performed. Regions of interest were defined to the fractured and normal contralateral tibia. Activities in regions of interest of both tibia sides in static images were counted. The intrapersonal ratios of the fractured and normal contralateral tibia sides in each rat were calculated.

Histological Evaluation

The specimens, which included the fracture line, were excised as en bloc. They were fixed in 10% buffered formaldehyde for 24 h and then decalcified in 10% formic acid solution at room temperature for 10 days. The decalcification solutions were changed every 3 days. Subsequently, the samples were dehydrated in graded ethanol, cleared in xylol, and embedded in paraffin. Using a microtome, 5-μm-thick longitudinal serial sections were taken from the specimens and stained with hematoxylin and eosin. All sections were examined under a light microscope (Olympus BX-51) and recorded by a histologist with no knowledge of the examination groups. The criteria described by Huo and his colleagues were used for the histological evaluation of the specimens [18]. In this system, the fracture callus was graded according to the relative amounts of fibrous tissue, cartilage, woven bone, and mature bone. According to this classification system, the following grade were achieved: Grade 1 indicated fibrous tissue; Grade 2 indicated predominantly fibrous tissue with some cartilage; Grade 3 indicated equal amounts of fibrous tissue and cartilage; Grade 4 indicated all cartilage; Grade 5 indicated predominantly cartilage with some woven bone; Grade 6 indicated equal amounts of cartilage and woven bone; Grade 7 indicated predominately woven bone with some cartilage; Grade 8 indicated entirely woven bone; Grade 9 indicated woven bone and some mature bone; and Grade 10 indicated lamellar (mature) bone. The mean histological score was calculated for each group.

Comparisons among groups were performed using Kruskal–Wallis analysis of variance (Mann–Whitney *t*-test with Bonferroni correction as post-hoc test) for scintigraphic, radiological, and histological scores. Results of scintigraphic evaluation were cited as mean ±

TABLE 1

Radiological Results of the Groups

Radiological score	3rd week median (Min-max)	5th week median (Min-max)	P
Group 1	2 (2–3)	3 (2–3)	0.093
Group 2	2 (2–3)	2.5 (2–3)	0.241
Group 3 (Control)	1.5 (1–2)	2 (2–2)	0.056
KW Chi-square	6.61	8.07	
df	2	2	
P	0.037	0.018	

Notes. Fracture healing score was highest in group 1 compared to all other groups. There was a statistical significant difference between the groups both at the 3rd week ($P = 0.037$) and at the 5th week ($P = 0.018$), but there was no statistical difference between groups 1 and 2 in both the 3rd and the 5th week ($P = 0.523$, $P = 0.241$ respectively).

SD. Results of radiological and histological evaluations scores were cited as median (min-max) value. A P value 0.05 was regarded as statistically significant.

RESULTS

There were no bacterial deep wound infections in any of the rats that underwent the procedure. We did not observe cannibalism. No rat died throughout experiment.

A total of 38 rats was used to gain 36 rats which had standardized fracture configuration. Two rats, which did not have standardized fracture configuration, were treated, withdrawn from the study, and replaced by others.

Radiological Evaluation

Scores according to the Lane–Sandhu Scoring System were summarized in Table 1.

At the 3rd week, while fracture-healing score was highest in group 1, it was lowest in group 3. There was a statistically significant difference between the groups ($P = 0.037$).

At the 5th week, there was a statistically significant difference between the groups ($P = 0.018$). This difference emanated from group 1 but there was no statistical difference between groups 1 and 2 in both the 3rd and the 5th week ($P = 0.523$, $P = 0.241$, respectively).

Scintigraphic Evaluation

We have preferred to establish comparisons with the normal contralateral tibia, considering that the increased blood flow in the fractured tibia is part of the bone healing process and should not be exclude from the calculations, unless we were only interested in the actual bone metabolic activity. We elected for all counts to be taken in delayed phase (after 3 hours from the radionuclide injection) as most of the radionuclide has already been taken up by the metabolically active bone and the amount of circulating radionuclide is very small.

In this study, the scintigraphy showed the usual appearance of increased radionuclide uptake at the fracture site. All results showed that group 1 had a higher radionuclide uptake ratio than the other groups at the 3rd week ($P = 0.010$) (Fig. 1). At the 5th week, uptake ratios increased in groups 2 and 3; however, it decreased in group 1 (Table 2).

Histological Evaluation

In the histological evaluation, the highest scores were obtained from group 1 as compared to all other groups (group 1 = 4.5 (3–6); group 2 = 4 (4–5); group 3 = 3 (2–5)), although there was no statistical significant difference between the groups at the 3rd week ($P = 0.094$). Equal amounts of fibrous tissue and cartilage were observed in group 3 (control group); this was cartilage with some woven bone in group 1 (Fig. 2A-C).

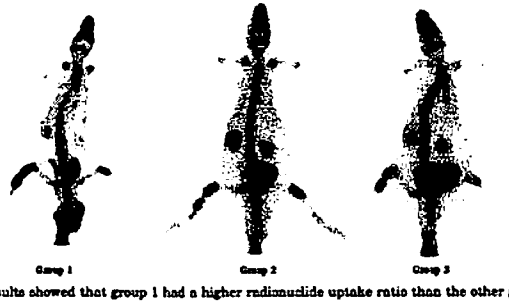

FIG. 1. Scintigraphic results showed that group 1 had a higher radionuclide uptake ratio than the other groups at the 3rd week.

TABLE 2

Scintigraphic Results of the Groups

Scintigraphic results	3rd week mean ± SD	5th week mean ± SD	P
Group 1	11.54 ± 4.04	6.09 ± 3.18	0.037
Group 2	6.78 ± 3.07	7.96 ± 2.27	0.522
Group 3 (Control)	4.83 ± 2.91	7.45 ± 2.23	0.055
KW Chi-square	9.21	1.63	
df	2	2	
P	0.010	0.444	

Notes. Scintigraphic results showed that group 1 had a higher radionuclide uptake ratio than the other groups at the 3rd week ($P = 0.010$). At the 5th week, it was also noticed that small increases in groups 2' and 3' uptake ratios still continued; however, it decreased in group 1.

TABLE 3

Histological Results of the Groups

Histological score	3rd week median (Min-max)	5th week median (Min-max)	P
Group 1	4.5 (3–6)	7 (6–8)	0.008
Group 2	4 (4–5)	6 (5–7)	0.008
Group 3 (Control)	3 (2–5)	5.5 (5–6)	0.006
KW Chi-square	4.72	6.66	
df	2	2	
P	0.094	0.036	

Notes. Although histological grading of the fracture healing in group 1 had highest scores both at the 3rd and at the 5th week, there was only statistical significant difference at the 5th week among the groups ($P = 0.036$).

Analysis in the 5th week revealed a statistically significant difference between groups ($P = 0.036$). Group 1 had highest scores (7 (6–8)), but this score was 5.5 (5–6) in group 3. In this period, although the results in group 1 were better than those in group 2, this did not revealed a statistical difference between group 1 and group 2 ($P = 0.127$). Histological results were summarized in Table 3. We observed predominant cartilage with some woven bone (Grade 5) in the histolog-

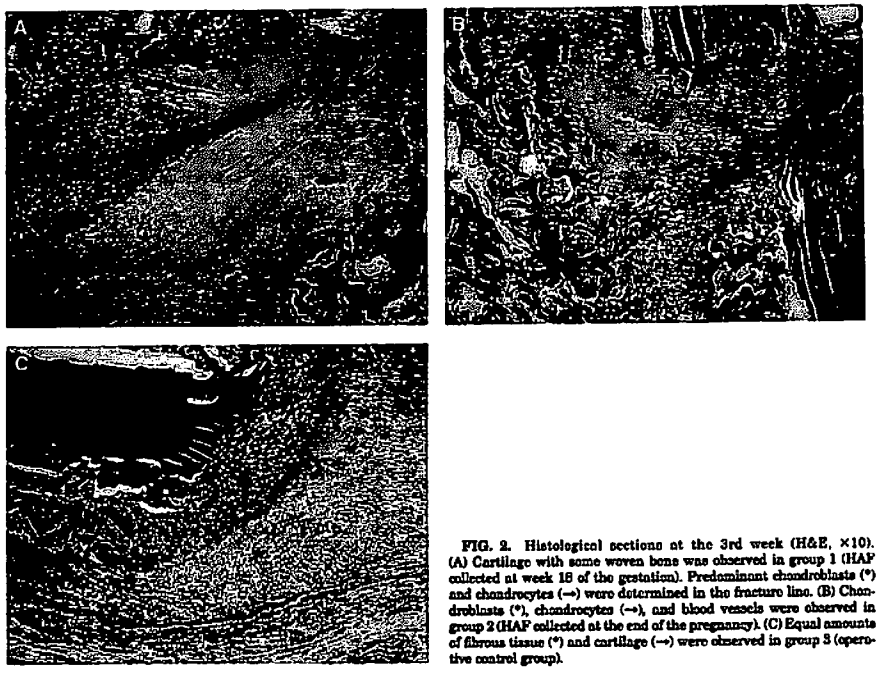

FIG. 2. Histological sections at the 3rd week (H&E, ×10). (A) Cartilage with some woven bone was observed in group 1 (HAF collected at week 18 of the gestation). Predominant chondroblasts (*) and chondrocytes (→) were determined in the fracture line. (B) Chondroblasts (*), chondrocytes (→), and blood vessels were observed in group 2 (HAF collected at the end of the pregnancy). (C) Equal amounts of fibrous tissue (*) and cartilage (→) were observed in group 3 (operative control group).

ical sections obtained from group 3. This was predominantly woven bone with some cartilage (Grade 7) in group 1 (Fig. 3A-C).

DISCUSSION

There are numerous cell-to-cell signaling peptides called growth factors which have positive effects on fracture healing. The sources of growth factors include the clot and the bone itself. These growth factors have an important role for bone remodeling and fracture healing [1, 2, 19, 20]. These factors have been identified at the site of the fracture including the TGF-β, FGFS, platelet-derived growth factor, IGF-I, and IGF-II [1, 2, 19]. During the past two decades, many studies have focused on TGF-β and FGFS, and it was reported that basic FGF is likely to play an important role in the initial phase of the fracture-healing process because of its angiogenic properties and mitogenic activity on the osteoblast lineage [1–4]. This factor regulates the expression of local regulatory factors in osteoblasts [21, 22] and also promotes fracture healing by the stimulation of bone remodeling [23]. In a study, Kawaguchi and coworkers reported that the application of recombinant human basic FGF increased the volume and mineral content of the calluses in a dose-dependent manner in both normal and diabetic rats [24].

The composition of the amniotic fluid includes many growth factors (FGF, EGF, IGF-I, and IGF-II), mucopolysaccharides (HA, HASA, chondroitin 4-, and 6-sulfate, dermatan sulfate, and heparan sulfate), and extracellular macromolecules (fibronectin and laminin). We thought that HAF including both growth factors and HA would have a positive stimulating effect on the fracture healing.

HA, which is a polysaccharide, has a positive effect on cell differentiation, migration, and invasion of various cell types. It provides therefore a mesenchymal signal for healing in bone, cartilage, nerve, and tendon [5, 10, 11, 25, 26]. It was reported that HA and HASA can be associated with accelerating new bone formation [10, 11].

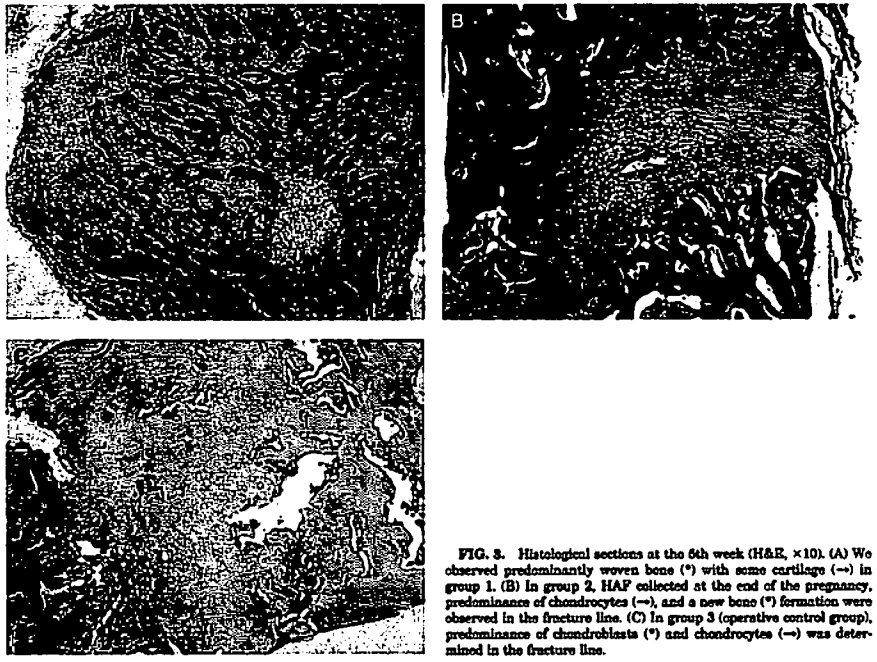

FIG. 3. Histological sections at the 5th week (H&E, ×10). (A) We observed predominantly woven bone (*) with some cartilage (→) in group 1. (B) In group 2, HAF collected at the end of the pregnancy, predominance of chondrocytes (→), and a new bone (*) formation were observed in the fracture line. (C) In group 3 (operative control group), predominance of chondroblasts (*) and chondrocytes (→) was determined in the fracture line.

In previous experimental studies, HAF was used to prevent peritendinous adhesion formation, bone defect healing, nerve regeneration, and cartilage regeneration due to content of HA, growth, and trophic factors [5, 10, 11, 25, 26]. Ozgenel and coworkers reported that injection of HAF underneath the free perichondrial grafts promotes the proliferation and differentiation of the chondrocytes [5]. In addition, it was reported that a single application of HAF is effective in the treatment of bone healing [10] and in preventing adhesion formation together with facilitating the tendon-healing process [26]. Our results showed that HAF enhanced the facture healing similar to the other experimental studies that were mentioned above. Both group 1 and group 2 had better results than group 3 (operative control group).

The mean concentration of HA is approximately 20 μg/mL at weeks 16–20 of the gestational period. It drops to approximately 1 μg/mL at week 30 and is then constant until end of the pregnancy [8]. Similarly it was reported that concentrations of some growth factors in HAF changed throughout gestation [6, 7]. Hofmann and Abramowicz reported that EGF concentrations of HAF were 35 ± 8 pM at weeks 15–22 of gestation, while these concentrations were 87 ± 71 pM at weeks 35–39 of gestation [15]. Similarly, Varner *et al.* reported that EGF levels in HAF were increased near term [12]. Because the concentration of HA and growth factors in HAF varies considerably during gestation, we used HAF obtained from two different time periods to compare the possible effects on fracture healing of these two different HAFs. Group 1 had better radiological, histological, and scintigraphic results than group 2, but no statistical difference was observed between these two groups according to all evaluation methods at the 3rd and the 5th week except scintigraphic results at the 3rd week.

In the scintigraphic evaluation, metabolic activity at the fracture site is an indicator of the fracture-healing status. There are generally three phases identified scintigraphically when evaluating fractures. While in acute and subacute phases, a gradual increase in metabolic activity on the fractured site occurs; in healing phase, a gradual decline in activity occurs over time. In our study, metabolic activity at the fracture site was observed in group 1 more than the others at the 3rd week. This result may be emanating from increased angiogenesis. This result indicated to us that osteogenesis developed more quickly than the others. In the 5th week, we believe that the osteogenesis still continued in group 2 and 3 due to the observed small increase in their uptake ratios. With this we believe that the observed decrease in the uptake ratio in group 1 could be a sign that remodeling had started. Because from the moment when ossification begins to when remodeling is taking place, there is a marked decrease in the metabolic activity of the bone-healing process [27].

Although fracture healing was better in both group 1 and group 2 than group 3 histologically, the best results were obtained from group 1 at the 3rd and 5th week. We think that this result can emanate from variable concentrations of the growth factors and HA throughout gestation.

Going by the fact that the results from the HAF collected at 18th week of the gestation were better, we believe that with a more concentrated form of these factors and polysaccharide, it is possible to increase the positive effects on fracture healing.

Recently pluripotent cells were isolated from the human and rodent amniotic fluid [28]. These cells, termed amniotic fluid-derived stem cells, were shown to give rise to adipogenic, osteogenic, myogenic, endothelial, neurogenic, hepatic, and chondrogenic lineages [29]. In our study, we used cell-free centrifuged HAF for evaluation of effects alone of the mucopolysaccharides and the growth factors in the HAF. Thus the present study was not affected by amniotic fluid-derived stem cells. In addition, we think that this valuable fluid may be stored in deep freeze in this manner and may be used as an allograft without any risk of reaction and infection.

We arrived at the conclusion HAF had a positive effect on fracture healing in rat tibia and also this positive effect was observed more in group 1 (HAF collected at 18th week of the gestation). Additionally further studies are also needed to assess the roles of the various growth factors of HAF in the fracture healing and to investigate the dose response curves for HAF in the fracture healing.

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