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Original article

Effect of hyaluronic acids as chondroprotective in experimental model of osteoarthrosis☆,☆☆

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ABSTRACT

Objective: to analyze the effects of hyaluronic acid of different molecular weights in an experimental model of osteoarthritis in rabbits.
Methods: forty-four male California rabbits were divided randomly into three groups and underwent resection of the anterior cruciate ligament in his right knee. After three weeks of the surgical procedure began three weekly intra-articular injections of hyaluronic acid native (Polireumin®)-PR, hyaluronic acid branched chain (Synvisc®)-S and 0.9% saline-P. All animals were sacrificed after twelve weeks of surgery and tibial plateau infiltrated the knees were dissected. Histological cartilage of the support areas of the tibial plateaus were stained with Alcian Blue pH 1.0, Alcian Blue pH = 2.5 and toluidine blue for research on the amount of proteoglycans. The intensity of staining was quantified on a Zeiss microscope apparatus Imager Z2 MetaSystems and analyzed by software MetaferMsearch.
Results: the effect of chondroprotetor hyaluronic acids used in the study was confirmed when compared to the control group, but the comparison made between them, there was no statistically significant difference regarding chondroprotetion.
Conclusion: the hyaluronic acids tested had chondroprotective effect, with no statistical difference with regard to the different molecular weights.

Efeito dos ácidos hialurônicos como condroprotetores em modelo experimental de osteoartrose

Palavras chave:
Osteoartrose
Ácido hialurônico
Ligamento cruzado anterior

RESUMO

Objetivo: analisar os efeitos do ácido hialurônico de diferentes pesos moleculares em modelo experimental de osteoartrose em coelhos.
Métodos: foram alojados de modo aleatório 44 coelhos da raça California, machos, em três grupos (PR, S e P) e submetidos a ressecção do ligamento cruzado anterior do joelho direito.

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Joelho
Coelhos

Decorridas três semanas do procedimento cirúrgico iniciaram-se as três injeções intra-articulares semanais de ácido hialurônico nativo (Polireumin®)-PR, ácido hialurônico de cadeia ramificada (Synvisc®)-S e soro fisiológico 0,9%-P. Todos os animais foram sacrificados após 12 semanas do ato cirúrgico e os platôs tibiais dos joelhos infiltrados foram dissecados. Cortes histológicos da cartilagem das áreas de apoio com maior espessura dos platôs tibiais foram corados com Alcian Blue pH = 1,0, Alcian Blue Ph = 2,5 e Azul de Toluidina para pesquisa da quantidade de proteoglicanos. A intensidade de coloração foi quantificada em um aparelho de microscopia ZeissImager Z2 Metasystems e analisada pelo software MetaferMsearch. A análise estatística consistiu no uso dos testes Kolmorogov–Smirnov, análise de variância (Anova), *t* de Student e qui-quadrado. O nível de significância usado foi de 5%.

Resultado: o efeito condroprotetor dos ácidos hialurônicos usados no estudo foi demonstrado quando comparado ao do grupo controle, porém, feita a comparação entre si, não houve diferença estatística quanto à condroproteção.

Conclusão: os ácidos hialurônicos testados obtiveram efeito condroprotetor, sem diferença estatística com relação aos diferentes pesos moleculares.

Introduction

Osteoarthrosis (OA) is the most common joint disease worldwide, with prevalence greater than 10% after the age of 50. This condition exhibits cartilaginous histological changes, and can result in significant functional limitation.[1,2] OA is the result of several factors in joint dysfunction and is characterized by cartilaginous degeneration and simultaneous bone, cartilage and connective tissue proliferation.[3] Among various treatment modalities currently available, the treatment with intra-articular injections of hyaluronic acid (HA) has shown beneficial effects in controlling the symptoms of knee OA.[4]

HA, a polysaccharide of the glycosaminoglycan family, contributes to the homeostasis of the normal articulation, showing lower concentration and decreased molecular weight in the synovial fluid in joints with osteoarthrosis.[5,6] HA administered in the form of intra-articular injections may enhance the regenerative effects of endogenous HA on joint cartilage, restore the viscoelasticity of the synovial fluid, contributing to the synthesis of HA by synoviocytes, and prevent the degradation of proteoglycans and collagen fibers present in the extracellular matrix. HA stimulates the metabolism, prevents apoptosis of chondrocytes, and inhibits chondral degradation and articular inflammatory responses.[6] These effects of therapy with the use of HA are attributed not only to its ability to alleviate the symptoms related to osteoarthrosis, but also to its interference in the progression of joint degeneration.[5–7]

Considering the scope and implications of the knee OA, nowadays we understand the importance of diagnosis and treatment in its early stages, so that its consequences are minimized.[8] So far, there is no interventions capable of inhibiting its evolution; hence, are essential options that allow reducing its progression. Intra-articular injections of different types of HA could be used for this purpose.

To evaluate the effects of these substances in gonarthrosis, in this research we proposed the use of an experimental OA model that resembles that mimics the condition in humans. The section of the anterior cruciate ligament (ACL) of the rabbit knee, the "stifle joint" (term used in veterinary anatomy for the joint similar to human knee in small animals, such as rabbits and dogs), mimics the morphological and biochemical changes observed in human osteoarthrosis, which allows the accurate reproduction of the results.[9,10]

The aim of this study was to evaluate the effect of intra-articular injections of native HA (Polireumin®, TRB Pharma, São Paulo, Brazil) and of branched-chain HA (Synvisc®, Novartis, São Paulo, Brazil), separately and comparatively between themselves, in OA induced by ACL section of rabbit knees.

Materials and methods

This experiment was conducted at the bioterium of the post-graduate course of Federal University of Paraná (UFPR). The Research Ethics Committee of the Department of Health Sciences, UFPR, evaluated and approved the research protocol of this study (registry CEP/SD: 001.004 SI 06-06).

Forty-four male California rabbits, which were kept before and during the procedures in cages (two animals per cage) in the unit bioterium, were used. The ration was standardized and the animals received water *ad libitum*. The rabbits were kept under controlled light (light–dark cycle of 12 h), with temperature (22 ± 1 °C), humidity and noise level kept stable, with an average weight of 3.5 kg. Initially, all animals underwent resection of the ACL. The right knee has been chosen just to standardize the experiment.

The surgical procedure consisted of a pre-operative anesthesia with 10 mg/kg of ketamine hydrochloride (Dopalen®) and 50 mg/kg of xylazine hydrochloride (Anasedan®), administered in the same syringe by intramuscular (IM) injection in the semimembranosus and semitendinosus muscle bellies of the right hind limb. On the same occasion, the rabbits were treated with an injection of penicillin 14,400 IU and streptomycin 6 mg (Pentabiotic Veterinary Reinforced® – Eurofarma) as antibiotic prophylaxis, and Flunamine® (Bayer) 2.2 mg/kg IM, as post-operative analgesia.

The right knee was subjected to trichotomy and antisepsis with polyvinylpyrrolidone (Povidini®). After application of sterile fields, a medial parapatellar incision was made in the

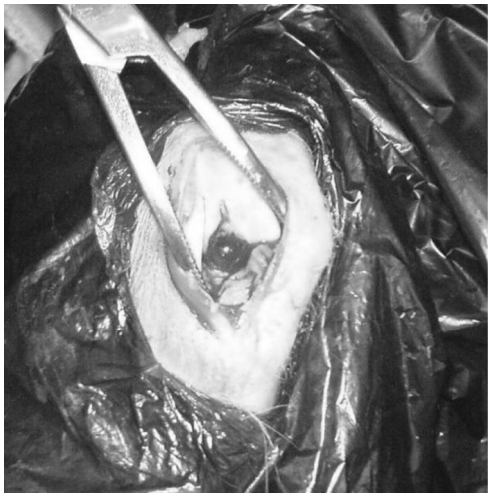

Fig. 1 – Photo of the skin incision and ACL capsulotomy.

skin and subcutaneous tissue, followed by capsulotomy and lateral dislocation of the patella (Fig. 1). Then, the knee was placed in maximum flexion, allowing visualization of the anterior cruciate ligament. This structure was sectioned with an scalpel blade nr. 15 (Fig. 2), the joint was irrigated with normal saline; and a capsulorrhaphy and skin suture with 4.0 monofilament nylon completed the operation (Fig. 3).

One rabbit in Group 2 and one in Group 3 developed infection of the surgical site with articular extension and were excluded from the study.

After surgery, the rabbits were kept in their cages without restricting the support to operated members. The animals were randomized into three groups, with 14 animals in each group. After three weeks of the surgical procedure, the animals were initiated in their intra-articular injections. The amount of hyaluronic acid was 0.3 ml, similar to the volume used in small joints of humans. Group P: control group, three injections (at weekly intervals) of 0.9% isotonic saline; Group PR: three injections (at weekly intervals) of native hyaluronic acid (Polireumin®); Group S: three injections at weekly intervals of branched-chain hyaluronic acid (Synvisc®).

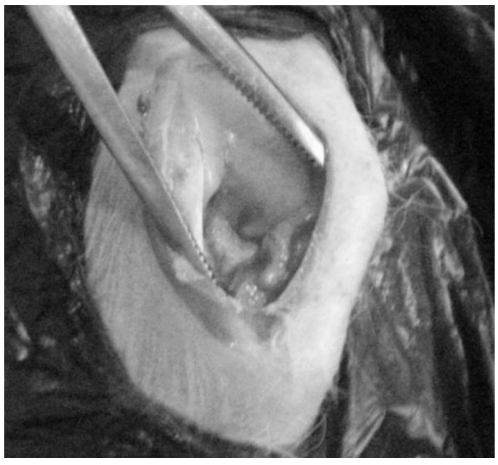

Fig. 2 – Photo of ACL exposure.

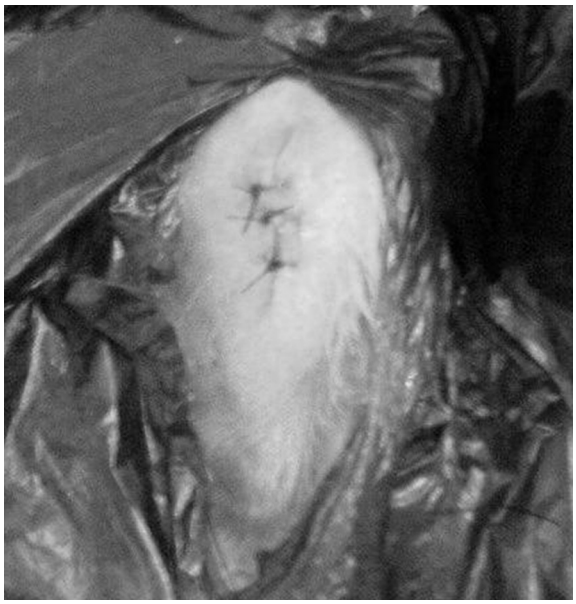

Fig. 3 – Photo of skin suture.

The rabbits were euthanized after 12 weeks of surgery; the animals were anesthetized as described previously and subjected to intracardiac injection of thiopental (5 mL) and potassium chloride (10 mL). The tibial plateaus were resected aseptically and immersed in a flask containing 10% formaldehyde. The vials were labeled for identification of groups and sent to the Clinical Pathology Service, Hospital de Clínicas.

The tibial plateaus were decalcified. The medials in the area of greater cartilage thickness were subjected to microtomy in the sagittal plane; then, three slides were prepared by tibial plateau, with the use of stains Alcian Blue pH 1.0, Alcian Blue pH 2.5, and Toluidine Blue and included in paraffin.

The stained slides were sent to the Polytechnic Center of the Life Sciences Sector, Federal University of Paraná.

The histological slides were automatically scanned into a ZeissImager Metasystems Z2 microscope, using the software MetaferMSearch with post-assembly with VSlide. Then, the cartilaginous regions were selected with the MetaViewer software snapshot tool. The pictures were analyzed by ImageJ software, using the RGB Stack tool for Alcian Blue pH 1.0, Alcian Blue pH 2.5 and the Color Deconvolution "RGB" for Toluidine Blue stains. Subsequently, the percentage of marked area was calculated after a threshold, followed by quantification.[11]

All study substances were acquired with own resources of researchers, without external financial support.

Data obtained were statistically analyzed by analysis of variance for single factor (ANOVA), followed by F and T unpaired tests, with significance when $p < 0.05$. Graphs were plotted with the mean standard deviation, using the software Microsoft Excel 2007. The data evaluation was objective (standardized by computer), and subjective, by scores given by the biologist responsible for the quantification of the staining in chi-square test. The scores 1, 2 and 3 were classified as poor, intermediate and excellent.

Table 1 – Numbers of quantification of Toluidine Blue.

Slides	Treatment groups		
	P	PR	S
1	66.81098	72.71096	96.52204
2	15.6544	50.25438	97.83475
3	48.30333	93.63707	73.56667
4	72.81427	98.97833	64.13119
5	76.35281	90.30573	49.14048
6	83.84236	73.03511	56.87234
7	30.08729	96.62571	66.2721
8	37.47977	73.87755	46.77349

Results

The decalcified slides, stained with Alcian Blue pH 1.0, Alcian Blue pH 2.5, and Toluidine Blue, showed the presence of glycosaminoglycans in larger quantities in the groups PR (Polireumin®) and S (Synvisc®) *versus* control group P (placebo). The significance level for all analyzes was 5%.

The evaluation of the staining intensity of histological sections stained with Alcian Blue pH 1.0, Alcian Blue pH 2.5 and Toluidine Blue with ZeissImager Z2 Metasystems microscopy generated results that were shown in tables and graphs. Below are graphs provided by the program, representative of the actual staining intensity measured in each group of the above-mentioned dyes.

Through the analysis conducted, each slide generated a datum in the set of data for each group: "P", "PR", or "S", totaling 24 slides/analyzed individuals (eight for each group). For the statistical analysis, the null hypothesis (H_0) was: there is no difference between the independent variables, and the alternative hypothesis (H_1) was: There is difference between the independent variables. An alpha value of 0.05 was used.

The values for each coloration are presented in Tables 1–4. The Kolmorogov–Smirnov test found a normal distribution of data (not shown). Thus, parametric tests were used: an analysis of variance (ANOVA) for single factor and unpaired Student *t*-test assumed equal variances. In the *F*-test, it was found that the variances of the groups did not differ within the same stain. In Toluidine Blue and Alcian Blue pH 2.5 (but not for pH 1.0) stains, ANOVA indicated a statistically significant difference ($p < 0.05$) (data not shown). Thus, for these two dyes with difference in ANOVA, Student's *t*-tests were performed. For Toluidine Blue staining, a difference was noted only between P *versus* PR groups (Table 2). For Alcian Blue pH 2.5, a difference was observed between P *versus* PR and P *versus* S groups, but the latter divergence was perceived only in the one-tailed analysis (Table 5). In order to verify whether there were a difference between the values of P and S groups, non-parametric analysis for categorical variables was performed. For this purpose, instead of analyzing the slides using the ImageJ software, scores from 1 to 3 for staining intensity were attributed, that is, the higher the score, the more intense the staining with Alcian Blue pH 2.5, as described above.[12]

The statistical test used was the contingency chi-square (χ^2) test. For this test, H_0 is: P and S groups did not differ in the pattern of staining intensity, while in $H_{1'}$ P and S groups

Table 3 – Numbers of quantification of Alcian Blue pH 1.0.

Slides	Treatment groups		
	P	PR	S
1	34.34797	68.02167	95.38368
2	89.68194	29.72821	97.26896
3	50.03834	81.88278	41.70293
4	41.21177	35.19409	68.65967
5	30.09164	34.54938	99.88211
6	38.7721	55.28347	44.2001
7	10.06779	43.21785	36.21376
8	32.54817	40.86236	29.08637

Table 4 – Numbers of quantification of Alcian Blue pH 2.5.

Slides	Treatment groups		
	P	PR	S
1	49.86103	61.96043	36.31462
2	37.0048	99.59628	96.2119
3	90.00677	89.87066	94.47586
4	39.21933	38.10635	63.63671
5	41.19301	99.1222	64.21882
6	35.90056	62.46105	60.2876
7	12.16039	72.56529	64.85414
8	28.92869	53.91113	29.84288

Table 2 – Analyses of Table 1 data with Student's *t*-test.

Information	Comparisons 2 × 2					
	P	PR	P	S	PR	S
Mean	53.9	81.2	53.9	68.9	81.2	68.9
Variance	607	277.4	608	382.3	277.4	382.3
Observations	8	8	8	8	8	8
Pooled variance	442.7		495.1		329.8	
H_0 difference	0		0		0	
Df	14		14		14	
t-Calculated	−2.6		−1.3		1.3	
P ($T \leq t$) tailed	0.01		0.10		0.10	
t-Tailed critical	1.8		1.8		1.76131	
P ($T \leq t$) two-tailed	0.02		0.20		0.20	
t-Two-tailed critical	2.1		2.1		2.1	

Table 5 – Analyses of Table 4 data with Student t test.

Information	Comparisons 2 × 2					
	P	PR	P	S	PR	S
Mean	41.8	72.2	41.8	63.7	72.2	63.7
Variance	499.7	497.8	499.7	559.1	497.8	559.1
Observations	8	8	8	8	8	8
Pooled variance	498.7		529.4		528.4	
H_0 difference	0		0		0	
Df	14		14		14	
t-Calculated	−2.7		−1.9		0.7	
P ($T \leq t$) tailed	0.01		0.04		0.2	
t-Tailed critical	1.8		1.8		1.8	
P ($T \leq t$) two-tailed	0.02		0.08		0.5	
t-Two-tailed critical	2.1		2.1		2.1	

Table 6 – Analysis of categorical data of Alcian Blue pH 2.5.

Treatment groups	Notes		
	1	2	3
P ($n = 8$)	62.5% (5)	12.5% (1)	25% (2)
S ($n = 8$)	25% (2)	12.5% (1)	62.5% (5)

Note: χ^2 calculated = 32.14286.

differ in the pattern of staining intensity. N = 16 (eight for each treatment group), alpha = 0.05, degree of freedom = 2, and tabulated $\chi^2 = 5.991$. χ^2 calculated was = 32.14286 (Table 6). As the calculated value of χ^2 is greater than the tabulated value, we reject H_0 and accept the alternative hypothesis.

Discussion

In recent decades, studies comparing the effectiveness of hyaluronic acids of different molecular weights have been published. The data are discrepant because of its results and methods of evaluation.[13] In clinical practice, the orthopedic surgeons have preferred the hyaluronic acid of high molecular weight for the treatment of osteoarthrosis, based on studies such as Atamaz et al.[14] and Wobig et al.[15] who used the model of osteoarthrosis in humans, compared hyaluronic acids of different molecular weights with saline intra-articularly infiltrated, and obtained better results with the use of hyaluronic acid of higher molecular weight by clinical and non-histological criteria.

However, according to Karlsson et al.[16] who studied hyaluronic acids of different molecular weights injected by intra-articular infiltrations in humans with osteoarthrosis, there was no significant difference among hyaluronic acids of different molecular weights, concerning clinical and non-histological criteria.

These controversies have led to the realization of this study, that compared the effect of a high molecular weight (Synvisc®) with a low weight (Polireumin®) hyaluronate. For its realization, we used an experimental model of osteoarthrosis.

The experimental model in small animals which most closely resembles the osteoarthrosis present in human beings is the transection of the anterior cruciate ligament in rabbit knee. This model mimics the morphological and biochemical changes observed in humans with osteoarthrosis.[5] The rabbit was the animal chosen because of its easy handling, lower cost, and the vast literature that confirms its use for the purposes of obtaining induced osteoarthrosis. The study period of 12 weeks is relatively short for obtaining AO, but this average time is used in most published studies in the literature. The anatomy of the rabbit knee is easy to dissect and allows visualization of the anterior cruciate ligament, which facilitates the surgical procedure of its transection.[9,10,17,18]

In the present study, the handling of the animals and the surgical dissection of the rabbit knees were easy to perform. The skin incision, the medial capsulotomy with visualization of the anterior cruciate ligament, and its transection had been done quickly and in a practical way. The knee plateaus undergoing this procedure showed signs of obvious macroscopic lesions, especially those in the control group P (placebo – 0.9% saline).[19]

Specific histological colorations for glycosaminoglycans were chosen to assess joint cartilage, because of their high sensitivity for detection of proteoglycans. Alcian Blue pH 1.0, Alcian Blue pH 2.5 and Toluidine Blue have great ability to stain glycosaminoglycans and proteoglycans, having been used successfully in several histological studies of cartilage.[20–23] The Alcian Blue pH 1.0 stains generally glycosaminoglycans in different tissues, whereas Alcian Blue pH 2.5 and Toluidine Blue specifically stain proteoglycans.

The use of the ZeissImager Z2 Metasystems microscope and of the MetaferMsearch software allowed the measurement of the staining intensity in the slides of cartilaginous tissue.

Greater staining intensity was obtained In S (Synvisc®) and PR (Polireumin®) groups *versus* control group P (placebo – 0.9% saline); hence, the decalcified and stained slides with Alcian Blue pH 1.0, Alcian Blue pH 2.5 and Toluidine Blue showed the presence of glycosaminoglycans in greater amounts, when compared with the control group P (placebo). This finding confirms the hypothesis that the hyaluronic acids tested act as chondroprotective factors, as they preserved a greater amount

of proteoglycans in the cartilaginous tissue of the knees infiltrated with hyaluronates.

There were no statistical differences in the intensity of staining of slides of knees treated with infiltration with high molecular weight *versus* low molecular weight hyaluronic acid. The transposition of the data found in rabbits in this study may not reflect the same findings in humans.

At the same time, an evaluation of the staining intensity by direct vision of the slides, that is, dependent on the examiner, was done. In this sense, we asked a biologist to assess the slides, ascribing scores. This professional rated with 1, 2, and 3 points the slides stained with Alcian Blue pH 2.5 as poor, intermediate and excellent, respectively. The results by direct vision confirmed the results conferred by the instrument, *i.e.*, there was no statistical difference between the groups treated with hyaluronates when compared with the placebo group, and there was no statistical difference between the groups infiltrated with hyaluronates, regardless of molecular weight. Subjective data were evaluated by chi-square test.

The literature that compares the different molecular weights of hyaluronates in this experimental model is scarce. Shimizu et al.[24] in a study in rabbits, concluded that hyaluronic acids of low molecular weight were superior *versus* those of higher molecular weight. Their study did not mention the dose used in the infiltration; hence we could not make a more appropriate comparison with our study. In our study, the rabbits were treated with intra-articular applications for three weeks (as is usually done in humans) and the volume used was 0.3 mL for the three substances tested (native hyaluronic acid, branched-chain hyaluronic acid, and normal saline). The half-life of native hyaluronic acid is 13 h, while branched-chain hyaluronic acid is 36 h. We chose a volume of 0.3 mL, because this is the recommended dose for use in small joints of humans.[25]

Ghosh and Guidolin[26] using an experimental model of transection of the anterior cruciate ligament in dogs, obtained better results with hyaluronic acids of lower molecular weight. The same authors, in an *in vitro* study, found better results with the use of hyaluronic acid of higher molecular weight, which contradicted their studies in animals. This substance would be a better stimulator of the production of extracellular matrix components. This could be partly explained, because the hyaluronic acid of lower molecular weight would penetrate the extracellular matrix more easily, maximize its concentration, and promote its interaction with target cells in the synovium. Furthermore, there is evidence that the binding of molecules of hyaluronic acid with cellular receptors is dependent on the molecular weight.[12,13]

There are several hypotheses trying to explain the mechanism of action of hyaluronic acids in non-pathological articulations of humans. In one of them, hyaluronic acid would act as modulator, through the interaction with CD44 receptors present in synoviocytes, and would act biochemically in the joint and decrease the production of cytokines, prostaglandins and metalloproteinases.[27–30] Arguably, hyaluronic acid also retrieves the physiological properties of synovial fluid, decreases the pressure of the strength/weight, and improves the distribution of the weight incident on the joint. Therefore, hyaluronic acids play an important role in the mechanical effects of the joints.[31]

In summary, in this study the data confirm the findings of Karlsson et al.[16] who studied the effects of hyaluronates of different molecular weights in intra-articular infiltrations in humans with osteoarthrosis, and suggest no differences in the molecular weight of hyaluronic acids, with respect to chondroprotection.

Conclusion

The analysis of the effects of native and branched-chain hyaluronic acids in an experimental model of osteoarthrosis in rabbits demonstrated chondroprotective proprieties, when compared to the control group (0.9% saline). When native chain and branched-chain hyaluronic acids were compared, no statistically significant difference was perceived.

Conflicts of interest

The authors declare that there were no conflicts of interest.

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