Study of the Protective Effects of Quince (*Cydonia Oblonga*) Leaf Extract on the Histologic Structure and Microscopic Indices of Spermatogenesis Following Induction of Diabetes in Adult Rats

Davoud KIANIFARD^{1*}, Gholamreza VAF AEI SAIAH², Emad KHALILZADEH²

¹ Division of Histology, Department of Basic Sciences, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, IRAN

² Division of Physiology & Laboratory Animals Facility, Department of Basic Sciences, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, IRAN

*corresponding author: davoudkianifard@gmail.com

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Abstract

Various types of infertility are associated with uncontrolled hyperglycemia. Development of oxidative stress is one of the most important factors in the alteration of spermatogenesis in diabetic conditions. Testicular tissue is one of the sensitive organs to environmental damages. Natural antioxidants are considered as preventive and therapeutic strategies in cases of diabetic side effects. *Cydonia oblonga* leaf extract contains natural antioxidant compounds. The flavonoid compounds of the quince have strong antioxidant and immune-regulatory effects. According to little data about the protective effects of *cydonia oblonga* leaf extract on the structural alterations of testicular tissue following induction of diabetes, in this study protective aspects of this extract on the diabetic reproductive alteration were evaluated.

Cydonia oblonga leaf extract was gavaged in two doses of 250 and 500 mg/kg. The number of 56 adult rats were divided into seven groups consisted of control, extract treated control groups, diabetic, extract treated diabetic groups and metformin treated diabetic group. Eight weeks after induction of diabetes, the body and testicular weight were measured and microscopic and histomorphometric studies were done on tissue samples.

In control groups, the administration of extract had no effect on histomorphometric parameters. In extract treated diabetic groups, the mean of body weight, histologic parameters and spermatogenesis indices were improved in comparison to non-treated diabetic group.

The results of this study showed that, natural antioxidant such as *cydonia oblonga* leaf extract in certain doses could be effective in reduction of hyperglycemic side effects on target organs.

Keywords: *Cydonia Oblonga Extract, Diabetes, Rat, Spermatogenesis, Testicular tissue*

INTRODUCTION

The acute and chronic complications of diabetes are the major problems that occur in diabetic patients (Yanardag *et al.*, 2005). Increase of blood glucose levels leads to structural and functional changes in various target tissues and organs (Cai *et al.*, 2000). Induction of diabetes in male rats is associated with alteration in functions of reproductive system (Orth *et al.*, 1979). Diabetes is one of the most important risk factors for male

infertility. In this regard, *in vivo* experiments on adult rats indicated that, induction of diabetes is associated with cellular alterations in testicular microenvironment (Kianifard *et al.*, 2011).

Diabetes is a condition of increased oxidative stress and impaired energy metabolism. Recent experimental and clinical studies suggest that oxidative stress and production of reactive oxygen species (ROS) play a key role in the pathogenesis of both types of diabetes mellitus and subsequently

development of diabetes complications (Maritim *et al.*, 2003; Giacco and Brownlee, 2010). Herbal drugs have gained importance because of their efficacy and cost effectiveness in treatment of several diseases. Flavonoids can inhibit the secretion of inflammatory mediators such as nitric oxide, interleukin XII and tumor necrosis factor alpha (Nijveldt *et al.*, 2001; Ostrowska and Skrzydlewska, 2005; Rao *et al.*, 2005). Some studies mentioned the reduction of oxidative reactions following the administration of flavonoids (Ostrowska and Skrzydlewska, 2005; Kurin *et al.*, 2012). Moreover, it has been demonstrated the flavonoid compounds of the quince have strong antioxidant and immune-regulatory effects (Nijveldt *et al.*, 2001). According to the growing prevalence of diabetes and to the importance of normal fertility in physical and mental health, the aim of this study was to evaluate the protective effects of quince leaves extract, as herbal antioxidant, on the structural and functional alterations of testicular tissue following induction of diabetes.

MATERIAL AND METHODS

Animal Procedure: The number of 70 adult male *Wistar* rats with (mean $\pm$ SD) of body weight 194.3 $\pm$ 4.34 g was used in this study. The animals were used for all testing, housed under a 12 hrs. light-dark cycle with room temperature of 23-25°C and had access to food and water ad libitum. All animal procedures used in this study, were approved by the *University of Tabriz* standards for human care and use of laboratory animals in accordance with the Research Ethical Committee of the Ministry of Health and Medical Education of Iran (adopted in April 17, 2006) based on the Helsinki Protocol (Helsinki, Finland, 1975).

Experimental Design: The animals were divided into seven experimental groups. Each group consisted of ten animals (two animals were considered for replacement in cases of animal loss in duration of study). At the end of study, eight rats were used for sampling. 1) *Control group (Cont.)*: normal and healthy rats that did not receive any type of treatment; 2) *Low dose extract administrated control group (Cont+Ext250)* which received quince leaf extract with dose of 250 mg/kg body weight for eight weeks; 3) *High dose extract administrated control group (Cont+Ext500)* which received quince leaf extract with dose of 500 mg/kg body weight for eight weeks; 4) *Diabetic*

group (Diab.): in this group, diabetes was induced and the animals were euthanized eight weeks after induction of diabetes; 5) *Low dose extract administrated Diabetic group (Diab+Ext250)*: in this group, two weeks after induction of diabetes, quince leaf extract was administrated in low dose to diabetic animals for a period of eight weeks; 6) *High dose extract administrated Diabetic group (Diab+Ext500)*: this group consists of diabetic rats which received high dose of quince leaf extract for eight weeks after induction of diabetes. 7) *Metformin treated Diabetic group (Diab+Met)*: this group consists of diabetic rats which received metformin for eight weeks after induction of diabetes. The experimental period was performed two weeks after induction of diabetes.

Treatments and Chemicals: Streptozotocin, STZ, (Sigma, ST. Louis, MO, USA) was used for induction of diabetes. The STZ was dissolved in 0.1 M citrate sodium buffer (pH 4.5) and was injected intraperitoneally (45 mg/kg body weight) in overnight fasting animals. Diabetes was confirmed 48 hours after injection of STZ with an automated glucose analyzer device (Glucometer, On Call EZ, SD, USA). The animals with blood glucose levels above 200 mg/dl were considered diabetic and were used in this study (Venkateswaran and Pari, 2002). The animals of group 7, received daily freshly prepared metformin HCL (GLUCOPHAGE, Merck Sante s.a.s., LYON - FRANCE) dissolved in distilled water (100 mg/kg b.w.) by oral gavages method from two weeks after induction of diabetes to the end of study (Yanardag *et al.*, 2005). The quince leaf extract was prepared according to Rodrigues *et al* method (Rodrigues *et al.*, 2002).

Measurement of Body and Testicular Weight: At the beginning of study, the weight of each animal was recorded and then, the mean of body weight of experimental groups was recorded twice a week during the study. At the end of study, the animals were euthanized and the weight of right and left testes were measured.

Tissue Preparation and Histological Techniques: The testicular tissues were immediately fixed in 10% formaldehyde in buffered solution containing 54 mM NaH₂PO₄ and 28 mM Na₂HPO₄ (pH 7.4) and kept at 4°C. After 48 hours, the transverse section was made on the middle part of each testis and kept immersed in the fixative solution for the completion of tissue fixation. Then, formaldehyde-fixed samples were

embedded in paraffin and sliced with thickness of 6-7 micrometer and were mounted onto albumin-pre-coated glass slides. The mounted tissue samples were stained by the Hematoxylin and eosin method (H&E) for histological observations by light microscopy.

Morphometric Analysis: For morphometric assessment of seminiferous tubules, the slides were studied at 200× magnification. To get extra precise results, only the seminiferous tubules (STs) that sectioned transversely were studied and the shortest diameter of seminiferous tubules was considered for measurement. For every one animal, three microscopic slides from different parts of testicular tissue were prepared and 200 tubules were studied.

Evaluation of Spermatogenesis in Testicular Tissue: For estimation of spermatogenesis in testicular tissue, three different indices were used. Tubular differentiation index (TDI), repopulation index (RI) and spermiogenesis index (SPI). To determine the tubular differentiation index, the number of seminiferous tubules with more than three layers of germinal cells derived from type A of spermatogonia was calculated. To find out the repopulation index, the ratio of active spermatogonia (with euchromatic nucleus) to inactive ones (with dense heterochromatic nucleus) was evaluated. The ratio of the number of seminiferous tubules with spermatozooids to the empty tubules, were calculated for spermiogenesis index (Shetty *et al.*, 2000; Meistrich *et al.*, 2003).

Statistical Analysis: The obtained results were analyzed using the *GraphPad PRISM®*

software version 5.04 (GraphPad Software, Inc. USA). All data were reported as mean ± SD. The comparison of means between experimental groups was evaluated by using the one way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Differences were considered to be statistically significant if $P < 0.05$.

RESULTS AND DISCUSSION

Blood Glucose: Induction of diabetes was led to significant increase of fasting blood glucose levels ($p < 0.0001$) in all diabetic groups in comparison to control groups (Tab. 1). In this regard, treatment of diabetic groups with metformin was led to significant reduction of fasting blood glucose ($p < 0.0001$) in comparison to other diabetic groups. Moreover, the administration of quince leaf extract to control groups was not led to any noteworthy change in blood glucose levels.

Body Weight and Testicular Weight: Table 1 shows the mean of body weight and testicular weight in experimental groups. The administration of quince leaf extract, dose dependently was led to decrement of body weight in control groups. In contrast, the mean of body weight was increased in extract treated diabetic rats compared to non-treated diabetic rats. The highest weight loss was observed in metformin treated diabetic group. The mean of body weight in control and Cont+Ext250 groups was significantly higher than all diabetic groups ($p < 0.001$). Figure 1 shows the changes of body weight from the beginning to the end of study. As table 1 shows, the mean of testicular weight was reduced significantly in

Tab. 1. Mean of body and testicular weight and fasting blood glucose levels in experimental groups

	Body Weight (g)	Testicular Weight (g)	Blood Glucose (mg/dl)
Cont.	206.1 ± 14.61*	2.325 ± 0.19 *	96.50±7.52 *
Cont+Ext250	203.3 ± 7.05 *	2.213 ± 0.19 *	96.88±9.04 *
Cont+Ext500	194.3 ± 4.51	2.20 ± 0.22 *	92.75±13.60 *
Diab.	180.3 ± 19.98	1.87 ± 0.13	308±49.94
Diab+Ext250	181.6 ± 13.65	1.81 ± 0.18	253.4±57.02
Diab+Ext500	184.7 ± 10.98	1.75 ± 0.11	244.3±73.91
Diab+Met	179.5 ± 22.10	1.70 ± 0.15	169±33.20 ^β

Note: Body and testicular weight: * Significant difference in comparison to all diabetic groups. **Blood glucose:** * Significant difference in comparison to all diabetic groups; ^β Significant difference in comparison to diabetic groups. Data are presented as mean±SD.

diabetic groups in comparison to control groups ($p < 0.001$). In diabetic groups, the most reduction of testicular weight was observed in metformin treated diabetic group.

Fig. 1. Diagram of body weight gain during the period of study. The body weight measurement was carried out twice a week.

Cellular and Morphometric Analysis of Seminiferous Tubules: The mean of external diameter of seminiferous tubules was reduced slightly in control groups following administration of quince leaf extract (Tab. 2). In diabetic groups, the administration of quince leaf extract was lead to increase of external diameter of STs but these changes were not significant. This index was reduced significantly in diabetic groups in comparison to control groups ($p < 0.001$). There was no significant difference between metformin treated diabetic group and extract treated control groups. As table 2 shows, the mean of internal diameter of STs was reduced in diabetic groups but this change was not significant. Following induction of diabetes the mean of germinal

epithelium height was reduced in diabetic groups in comparison to control group ($p < 0.001$). Treatment of diabetic rats with quince leaf extract was lead to non-significant increase of height of germinal epithelium. Significant difference was observed in mean of germinal epithelium height between three diabetic groups and quince leaf treated control groups. Thickness of testicular capsule was increased in diabetic groups in comparison to control groups. In this regard, the most thickness of testicular capsule was observed in non-treated diabetic group (Tab. 2).

The mean of cellular population of STs Wall was summarized in table 3. Accordingly, the number of normal Sertoli cells was reduced in quince leaf extract control groups. The number of Sertoli cells was reduced significantly in non-treated diabetic and extract treated diabetic groups in comparison to control and Cont+Ext250 groups. Treatment of diabetic rats with metformin was lead to significant increment of Sertoli cells in comparison to other diabetic groups ($p < 0.001$). The mean of number of spermatogonia was not changed significantly in experimental groups. The number of primary spermatocytes was reduced in diabetic groups. In this regard, the administration of quince leaf extract dose dependently led to increase of primary spermatocytes in diabetic groups. In metformin treated diabetic rats the number of primary spermatocytes was significantly higher than non-treated diabetic group. The mean of number of round spermatids significantly reduced in diabetic groups in comparison to control groups ($p < 0.001$). Treatment of diabetic rats with quince

Tab. 2. Morphometric analysis indices in experimental groups.

	External Diameter (μm)	Internal Diameter (μm)	Germinal Epithelium height (μm)	Capsular Thickness (μm)
Cont.	429.8 $\pm$ 33.27 *	282.3 $\pm$ 32.95	141.5 $\pm$ 14.75 *	58.30 $\pm$ 7.21
Cont+Ext250	413.2 $\pm$ 24.52 $^{\beta}$	280.7 $\pm$ 30.69	135.6 $\pm$ 10.29 $^{\beta}$	56.10 $\pm$ 6.59 *
Cont+Ext500	415.5 $\pm$ 17.98 $^{\beta}$	275.7 $\pm$ 21.26	136.7 $\pm$ 11.36 $^{\beta}$	57.50 $\pm$ 6.41
Diab.	369.5 $\pm$ 44.19	257.9 $\pm$ 50.16	109.2 $\pm$ 20.28	67.40 $\pm$ 9.16
Diab+Ext250	372.6 $\pm$ 32.68	257.7 $\pm$ 38.79	115 $\pm$ 14.41	62 $\pm$ 6.92
Diab+Ext500	375.1 $\pm$ 34.39	260 $\pm$ 43.31	118 $\pm$ 16.52	61.40 $\pm$ 9.44
Diab+Met	394.8 $\pm$ 26.15	275.9 $\pm$ 24.62	122.3 $\pm$ 19.27	60 $\pm$ 6.89

Note: External Diameter & Germinal epithelium: * Significant difference in comparison to all diabetic groups; $^{\beta}$ Significant difference in comparison to all diabetic groups except metformin treated diabetic group. **Capsular thickness:** * Significant difference in comparison to diabetic group. Data are presented as mean $\pm$ SD.

Tab. 3. Cellular population of germinal epithelium of seminiferous tubules in experimental groups.

	Sertoli (#cell/10tubules)	Spermatogonia (#cell/10tubules)	Primary Spermatocyte (#cell/10tubules)	Spermatids (#cell/10tubules)
Cont.	429.8±33.27 [*]	282.3±32.95	141.5±14.75 [*]	58.30±7.21 [*]
Cont+Ext250	413.2±24.52 [*]	280.7±30.69	135.6±10.29 ^β	56.10±6.59 [*]
Cont+Ext500	415.5±17.98 ^β	275.7±21.26	136.7±11.36 ^β	57.50±6.41 [*]
Diab.	369.5±44.19	257.9±50.16	109.2±20.28	67.40±9.16
Diab+Ext250	372.6±32.68	257.7±38.79	115±14.41	62±6.92
Diab+Ext500	375.1±34.39	260±43.31	118±16.52	61.40±9.44
Diab+Met	394.8±26.15 ^α	275.9±24.62	122.3±19.27 ^α	60±6.89 ^α

Note: Sertoli cells: ^{*}Significant difference in comparison to all diabetic groups except metformin treated diabetic group; ^βSignificant difference in comparison to *Diab.* and *Diab+Ext500* groups; ^αSignificant difference in comparison to diabetic groups. **Primary Spermatocyte:** ^{*}Significant difference in comparison to all diabetic groups; ^βSignificant difference in comparison to all diabetic groups except metformin treated diabetic group; ^αSignificant difference in comparison to diabetic group. **Spermatids:** ^{*}Significant difference in comparison to all diabetic groups; ^αSignificant difference in comparison to diabetic group. Data are presented as mean±SD.

leaf extract led to increase of the number of spermatids. In metformin treated diabetic group, the number of spermatids was significantly higher compared to non-treated diabetic group.

Microscopic Indices of Spermatogenesis:

Tubular differentiation index (TDI) was reduced in diabetic groups in comparison to control groups (Fig. 2a). Moreover, treatment of diabetic rats with quince leaf extract led to increase of this index in comparison to non-treated diabetic rats. In metformin treated diabetic group, this index was non-significantly higher than other diabetic groups. The index of spermiogenesis (SPI) was higher in control groups in comparison to diabetic groups (Fig. 2b). In diabetic groups, treatment with quince leaf extract was led to improvement of this index but, this difference was not significant ($p > 0.05$). According to figure 2c, the most reduction of repopulation index (RI) was observed in non-treated diabetic group. Moreover, dose dependent administration of quince leaf extract to diabetic groups led to elevation of this index in comparison to non-treated diabetic rats.

Fig. 2. Microscopic indices of spermatogenesis. **2a and 2b:** ^αSignificant difference in comparison to diabetic group. **2c:** ^αSignificant difference in comparison to diabetic group; ^βSignificant difference in comparison to *Diab+Ext250* group; ^γSignificant difference in comparison to *Diab+Met* group. Data are presented as mean±SD.

Histology of Testicular Tissue: Figure 3 shows the histologic structure of testicular tissue in experimental groups. Tubular atrophy, disarrangement of germinal epithelium and

depopulation of spermatogenic cells was the most visible changes observed in diabetic groups. Moreover, above mentioned changes were reduced in quince leaf extract treated diabetic rats.

Fig. 3. Histologic structure of testicular tissue in experimental groups. A, B: *Control* group. General structure of STs with normal architecture (A); structure of normal ST with higher magnification (B). C, D: *Cont+Ext250* group. The structure of STs and germinal epithelium are similar to control group. E, F: *Cont+Ext500* group. Slight tubular atrophy is visible (1). G, H: *Diabetic* group. Increase of capsular thickness (2) and malformation of STs (3) is visible (G); reduction of spermatogenic cells (4) is visible (H). I, J: *Diab+Ext250* group. Tubular atrophy (5) is visible (I); germinal epithelium population is higher than diabetic group (J). K, L: *Diab+Ext500* group. Tubular atrophy (6) still visible (K); disarrangement and depopulation of germinal epithelium considerably decreased in this group (L). M, N: *Diab+Met* group. Mild tubular atrophy (7) is visible in some STs (M); arrangement and populace of spermatogenic cells somewhat is similar to control group (N). Red arrow: Sertoli cell; Yellow arrow: Spermatogonia; Black arrow: Spermatocyte; Blue arrow: Spermatid. (Hematoxylin & Eosin stain; A,C,E,G,I,K,M: 100 $\times$; B,D,F,H,J,L,N: 400 $\times$)

In this study the protective effects of quince leaf extract on the microscopic alterations of testicular tissue following induction of diabetes were investigated. Diabetic complications are the major problems that occur in diabetic patients (Yanardag *et al.*, 2005). Oxidative stress and production of reactive oxygen species (ROS) play a key role in the pathogenesis of diabetes mellitus and subsequently development of diabetes complications (Maritim *et al.*, 2003; Giacco and Brownlee, 2010).

Induction of diabetes is associated with cellular alterations in testicular microenvironment (Kianifard *et al.*, 2011). The Quince (*cydonia oblonga*) has a large amount of phenolic acids and flavonoids as antioxidant compounds (Silva *et al.*, 2004; Oliveira *et al.*, 2008; Magalhaes *et al.*, 2009). Reaction of flavonoids with free radicals leads to formation of more stable radicals with lower cytotoxicity. As well, they are capable of chelating iron ions (Fe^{2+}) which lead to reduction of side effects of free radicals (Nijveldt *et al.*, 2001). In this way, flavonoids can inhibit the secretion of inflammatory mediators such as nitric oxide, interleukin XII and tumor necrosis factor alpha (Nijveldt *et al.*, 2001; Ostrowska and Skrzydlewska, 2005; Rao *et al.*, 2005). Some studies mentioned the reduction of oxidative reactions following the administration of flavonoids (Ostrowska and Skrzydlewska, 2005; Kurin *et al.*, 2012). It has been demonstrated that, flavonoid compounds of the quince have strong antioxidant and immune-regulatory effects (Nijveldt *et al.*, 2001). Moreover, it has been demonstrated that the most phenolic contents have been found in leaves in comparison to other parts of this plant (Oliveira *et al.*, 2007). Flavonoids are the classes of compounds that have antidiabetic properties. Intake of flavonoids might prove to be important for alternative diabetes treatments or reduction of the risk of the disease (Coman *et al.*, 2012). In the present study, the mean of blood glucose was reduced in extract treated diabetic rats. Various phenolic compounds such as kaempferol have been reported for *cydonia oblonga* (Oliveira *et al.*, 2008). It has been found that kaempferol molecules protected the cells against the apoptosis induced by oxidative stress (Lee *et al.*, 2010). As a result, the flavonoid compounds through reducing of the pancreatic β -cell death can be consider as one of the possible mechanisms in cases of reduction of blood

glucose in quince leaf extract treated diabetic rats. According to our results, it seems that the ability of quince leaf extract in reducing of blood glucose levels is lower than metformin. In this regard, several factors such as the route of administration, the period of administration and the effective dose of extract can affect the hypoglycemic effects of quince leaf extract in comparison to metformin. In this study, treatment of control groups with quince leaf extract was lead to slight decrease of body weight while, in quince leaf extract treated diabetic groups increase of body weight were observed. According to figure 1, it seems that, diabetes related weight loss process is reduced following treatment of diabetic rats with quince leaf extract. The reduction of body weight can be due to breakdown of tissue proteins (Andulla and Varadacharyulu, 2003; Yanardag *et al.*, 2005). Diminution of pancreatic β -cells depopulation and improvement of glucose metabolism could be effective in weight gain of diabetic rats following the administration of quince leaf extract. It seems that, the changes of gonadal weight somewhat are similar to the changes of body weight. Our study is the first in-vivo report about the effects of quince leaf extract on the alterations of testicular tissue in diabetic conditions.

Excessive production of reactive oxygen species and microvascular damage are reported to substantially contribute to the occurrence of structural and functional changes (Nishikawa and Araki, 2007; Shrilatha and Muralidhara, 2007; Aitken and Roman, 2008). Disruption of spermatogenesis in diabetic rats was reported in various studies (Cai *et al.*, 2000; Guneli *et al.*, 2008; Kianifard and Rezaee, 2015). According to our results, in diabetic rats decrease in diameter of STs was accompanied with depletion in the height of germinal epithelium. This finding indicates that, the STs became atrophied during the course of diabetes. These histological observations in STs, illustrated depressed cellular activity of spermatogenic cells. Oxidative stress in testicular tissue has a direct relationship with abnormal spermatogenesis due to decrement of glutathione in male germ cells which leads to incomplete functional maturation and capacitation of spermatozoa (Maiorino and Ursini, 2005; Reddy *et al.*, 2006). Our study showed that, the administration of quince leaf extract, leads to

improvement of histomorphometry of testicular tissue in diabetic groups.

In our study, the number of normal sertoli cells (normal nuclear shape and position of cell) decreased in diabetic groups. This reduction could be due to depopulation of germ cells. In diabetic rats, the number of primary spermatocytes decreased significantly in comparison to control groups. This finding indicates that, the conversion of spermatogonia to primary spermatocytes is reduced in diabetic conditions.

The evaluation of microscopic indices of spermatogenesis confirms these histologic results. Diminished tubular differentiation (TDI) and spermiogenesis (SPI) indices in diabetic rats indicates that, conversion of spermatogonia to primary spermatocytes is reduced. Reduction of repopulation index in diabetic rats demonstrates the number of inactive spermatogonia increased after induction of diabetes. This process causes a decline of the number of primary spermatocytes derived from spermatogonia cells. These alterations in cellular conversion and/or activity lead to reduction in production of spermatozooids. It seems that, prevention of oxidative damage with natural antioxidants can be considered as one of the most effective strategies in control and reduction of diabetic complications in laboratory animals. The results obtained in this study, are valid for experimental diabetes and cannot be extrapolated automatically to human diabetes.

CONCLUSION

According to the results of this study, it seems that, quince leaf extract may play a positive role in improvement of microscopic structure of testicular tissue in diabetic conditions due to its antioxidant properties.

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