

Original Article

Effects of hypercholesterolemic diet by long-term on elastic system fibers penile tissue: volumetric density analysis of elastic system fibers

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Received October 10, 2015; Accepted November 24, 2015; Epub January 1, 2016; Published January 15, 2016

Abstract: The aim of the study was to assess the volumetric density of Elastic System Fibers (ESF) in the Corpus cavernosum (CC) of the penis of hypercholesterolemic rabbits by long-term. Fourteen New Zealand white rabbits were used. Hypercholesterolemia was induced at 6 months of age in 07 rabbits by oral. Two doses of 10 ml of chicken egg yolk offered at 7 am and 11 am (hypercholesterolemic group) for 24 weeks. The remaining 07 rabbits served as a control group. After 24 weeks, the rabbits were killed using sodium thiopental. Midshaft penile fragments were obtained and processed by routine histological techniques. Stereological analysis of ESF was performed in 5- μ m sections by using a M42 test grid system. Data were expressed as volumetric density (%) and Mann-Whitney U test was used and statistical significance was considered when $P < 0.05$. In the CC of hypercholesterolemic rabbits, the ESF were decreased by 38.6% ($P < 0.0006$). Penile tissue was affected by long-term of experimental hypercholesterolemia, possibly these changes could have an impact on penile structure during erection, and therefore might adversely affect erection maintenance.

Keywords: Rabbit penis, hypercholesterolemia, elastic fibers, erectile dysfunction, stereology

Introduction

Atherosclerosis is a kind of disease with multiple factors. Presently, inflammation is now well accepted to be the dominant factor in the pathogenesis and development of atherosclerotic diseases, where the hyperlipidemia is a major classical risk factor resulting in the development and progress of atherosclerosis [1].

Elevated plasma cholesterol concentrations induced by cholesterol feeding result in the development of atherosclerosis and impairment in endothelium-dependent vasodilatation in rabbits [2]. As well as, it has been demonstrated that this inflammatory disease induces two significant pathological processes: an ischemic event due to blood flow obstruction and vascular contractile dysfunction [3].

Longitudinal population-based studies clearly demonstrate that cardiovascular risk factors

such as dyslipidemia, central obesity, hypertension and insulin resistance are major risk factors for vasculogenic erectile dysfunction as well [4]. Furthermore, recent data suggest that the clustering of these factors, as occurs in patients with metabolic syndrome, increases the risk for the development of erectile dysfunction (ED) even further [5].

The erect penis has always been a symbol of power, virility and fertility. Erectile dysfunction is the consistent inability to achieve and maintain an erection sufficient for satisfactory sexual activity and may be the early clinical manifestation of a generalized vascular disease and carries an independent risk for cardiovascular events [6]. ED is associated with the presence and extent of asymptomatic atherosclerosis, including that of the coronary arteries, and precedes the development of clinically evident coronary artery disease (CAD) by a significant amount of time [5].

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Additionally, experimental evidence from different animal models applied to ED indicates that the morphological alterations in the penis may occur [7-11]. These changes could affect smooth muscle cells and different components of the extracellular matrix (ECM), including collagen and ESF. These are important penile components that maintain penile structure during erection, allowing adequate resistance during the return to the flaccid state [12, 13]. However, the way in which these various elements are affected is not yet well established, and precise quantitative data regarding ESF of the penis in by long-term hypercholesterolemia is not yet well reported in the literature. Thus, the present study aimed to evaluate the volumetric density of ESF in the *Corpus cavernosum* (CC) in the penis of long-term hypercholesterolemia rabbits.

Material and methods

The handling of the animals was approved by the Animal Care and Use Committee of Federal University of Alagoas, which based their analysis on the Guide for the Care and Use of Laboratory Animals and the study design was approved (N.011034/2007-55) by the local Ethics Committee for the care and use of laboratory animals.

Animals

Fourteen New Zealand (*Oryctolagus cuniculus*) male rabbits with a mean age of six months and a mean weight of 3 kg were included in this study. The animals were quarantined, examined and monitored by a veterinarian; housed in individual cages-all The rabbits were kept in a room with controlled temperature ($25\pm 1^{\circ}\text{C}$) and with artificial dark-light cycle (lights on from 07:00 hours to 19:00 hours).

Experimental model

These rabbits were divided into two groups according to diet. Standard rabbit feed (Purina®) (200 g/day) and water ad libitum was given to the 7 rabbits in group 1 (control group) for 24 weeks. In the group 2 (hypercholesterolemic group), the rabbits ($n = 7$) were fed the same diet of group 1, supplemented by 20 ml/day (4,32 g/day-cholesterol) of chicken egg yolk (carnauba®, Brazil) offered in twice a day for 24 weeks.

Blood sampling and analysis

Serum cholesterol/fractions were assayed on capillary blood (central ear vein) samples (Thermo Electron®-Brazil) from fasting animals and monitored at 0, 33, 66 and 99 days of dietary intervention. Rabbits were considered hypercholesterolemic when the cholesterol serum level was higher than $> 240 \text{ mg/dL}$.

Tissue specimens and histochemical analysis

Both groups were fed with the same standard pellets for rabbits and, 24 weeks after feeding protocol (G2), the rabbits were killed by an overdose (30 mg/kg) of sodium thiopental after subcutaneous 30 mg/kg xylazine combined with 9 mg/kg ketamine hydrochloride anesthesia.

The penises were dissected and a fragment of the middle shaft was removed and immediately fixed in phosphate buffered formalin solution (4% in 0.1 mol L⁻¹; pH 7.4) for 24 hours. Fragments of *Corpus cavernosum* (CC) were obtained and submitted to isotropic and random orthogonal triplet probe sections ("ortrip" cleavage) for stereologic analysis [14]. This method consisted of 3 random slice sections, the second section being orthogonal to the first and the third section also being orthogonal to the second. Thus, isotropic uniformly random sections were obtained [15].

After the "ortrip" cleavage the samples were processed for embedding in paraffin and sections of 5- μm thickness were obtained. To demonstrate the ESF the sections were stained with Weigert's resorsin fuchsin technique with previous oxidation [16, 17]. All samples were initially diagnosed by a pathologist (not a coauthor) to detect any foci of others pathologies and to exclude samples with artifacts.

Main outcome measures

From each penis, five different sections were selected from five fragments. From each section, five random fields were analyzed, totaling 25 fields (test areas) for each penis. The data were expressed as volumetric density (Vv-%). The analyzed fields were then digitized to a final magnification of $\times 400$ using a video camera coupled to a light microscope. The selected histologic areas were then quantified by applying a

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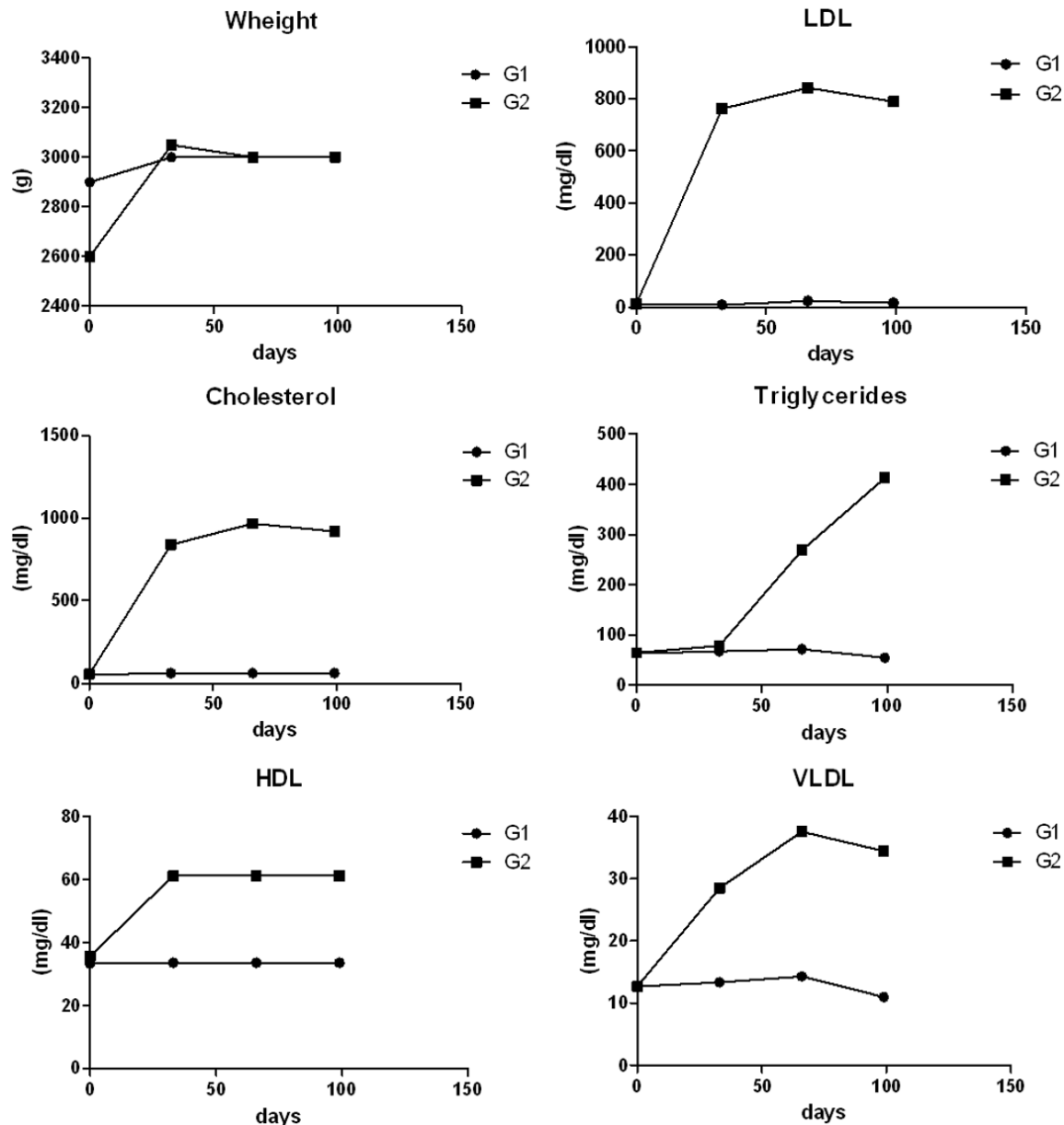


Figure 1. Results of blood cholesterol levels from rabbits submitted to hypercholesterolemia (G2) by egg yolk for a long-term compared with control group (G1).

test-grid system (M42 test-grid) on the digitized fields on the screen of a color monitor [15]. All numerical results are presented as mean $\pm$ standard deviation.

From stereologic principles in isotropic tissue, the area distribution of a given structure, as determined on a two-dimensional section of the structure, is proportional to the volume distribution of the structure. The volume density of the histologic components was calculated as $V_v = P_p/P_t$, where V_v was the volume density, P_p

was the tissue component under consideration, P_p was the number of test points associated with P , and P_t was the number of points in the test system. The stereologic methods have been described in detail elsewhere [15].

Statistical analysis

The data are reported as mean $\pm$ SD and the statistical significance was determined using the unpaired test (Mann-Whitney). The level of significance was set at $P < 0.05$. All statistical

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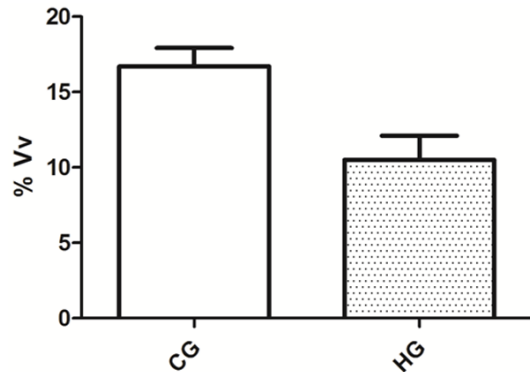


Figure 2. Results of amount (Vv%) of ESF in the Corpora cavernosa of the rabbit penis. G1 = Control group; G2 = Hypercholesterolemic group.

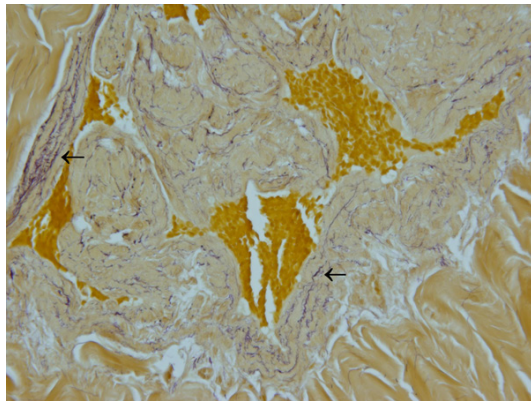


Figure 3. -ESF (arrows) in the CC of the penis from 48 weeks old rabbits. Representative images of control group (G1), stained with Weigert's resorsin fuchsin with previous oxidation and photographed at a final magnification of 200×.

analysis was done using GraphPad Prism 5 statistical software (GraphPad, San Diego, CA, USA).

Results

Blood sampling

Blood cholesterol concentration 33 d after experimental hypercholesterolemia had already approached a mean value of 838.39 mg/dL (**Figure 1**). Subsequently, this value increased steadily, with little variability among fractions, whereas, in controls, the blood cholesterol concentration had a constant mean value of ≈62 mg/dL throughout the duration of the experiment. When all animals were sacrificed, 24 weeks after treatment, blood experimental hypercholesterolemia was ≈919 mg/dL. Therefore, rabbits treated with chicken egg yolk

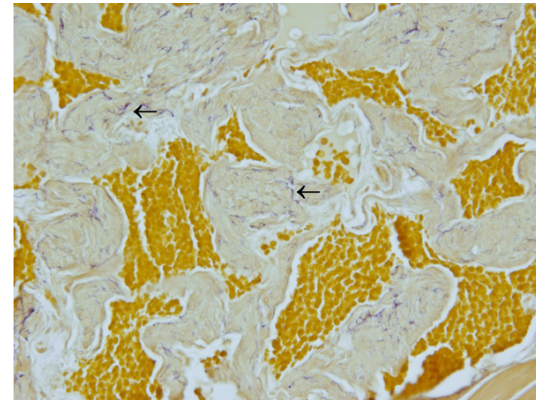


Figure 4. -ESF (arrows) in the CC of the penis from 48 weeks old rabbits. Representative images of Hypercholesterolemic group (G2), stained with Weigert's resorsin fuchsin with previous oxidation and photographed at a final magnification of 200×.

remained in a hypercholesterolemic condition for at least 20 weeks.

Figure 1 shows the body weight gain in the two groups (no statistically significant difference noted during the experiment), also may be observed all fractions cholesterol and triglyceride concentrations were significantly increased within four weeks after the start of the experiment and maintained until changed 99 d.

Morphological analysis

The rabbit has a vascular penis, which contains two erectile structures: a supero-lateral *Corpus cavernosum* (CC) and the ventral *Corpus spongiosum* (CS) surround the penile urethra. Both structures were covered by a dense capsule of connective tissue, the tunica albuginea (TA), which projects intracavernosal pillars or septa, mainly in the CC.

The histochemical analysis confirmed the presence of ESF in penile tissue of all specimens observed with an irregular network abundantly distributed throughout the CC (**Figure 3**). The volumetric density (Vv%) of the elastic system fibers in the penile tissue of G1 and G2 was 16.83%±1.22% and 10.33%±1.6%, respectively (differences statistically significant, $P \leq 0.0006$; **Figures 3, 4** and **Table 1**).

Discussion

Morphometric data

Area density has been used by many studies, attempting to quantify the ESF of urogenital

Table 1. Shows the results (Mean $\pm$ SD) of the two groups analyzed. The stereological quantification in the corpora cavernosa showed that the Vv of ESF was significantly lower (38.63%) in hypercholesterolemic rabbits (G2)

Results of ESF in Corpora cavernosa of the rabbit penis				
	Group 1	Group 2	n	P value
ESF (Vv%)	16.83 $\pm$ 1.22	10.33 $\pm$ 1.60	07	($\leq$ 0.0006)*

KEY: ESF = elastic system fibers. Data presented as the mean percentage $\pm$ SD. *Statistically significant, Mann-Whitney U test.

structures by using computer-aided image analysis software [12, 18]. These programs use the color property of the elements (pixels) of an image to determine a threshold level for inclusion. However, this method is limited to quantifying only thin and line-shaped structures [19].

Stereological methods have been used in quantification studies specifically to determine the number or proportion of fibrous components of the ESF [11, 17, 20-23]. The point counting method has proven to be very efficient in our study. It avoids the bias that frequently occurs with computerized image analyses, which may overestimate or underestimate the analyzed structures [19]. The stereologic methods have been described in detail elsewhere [15].

Experimental model

Studies with experimental models to develop atherosclerosis, claim that rabbits subjected to a hyperlipidemic diet with chicken egg yolk, developed vascular atherosclerotic injuries similar to those found in humans, considering, thus, dietary cholesterol as a precursor to the development of atherosclerosis [24, 25]. To our knowledge, the concentration of cholesterol observed in chicken eggs is 956 mg/100 g, presenting a high content of fat [25]. Thus, the chicken egg is considered important in significant increases in plasma cholesterol concentrations, with levels increased more than 1,000 mg/dl [26], and in the development of hyperlipidemia that, in turn, progress to atherosclerosis [24]. Therefore, the results of the experiment of this study (**Figure 1**) are reliable evidence indicating the importance of the type of fat in relation to the risk of cardiovascular disease, as well as erectile dysfunction; proving thus that a diet high in cholesterol and saturated fat has serious systemic metabolic consequences. The

levels of total cholesterol, LDL-c, VLDL-c, and triglycerides rapidly increase in the rabbit, especially in adult animals, thus developing the atherosclerosis process more easily than young rabbits. When starting an atherogenic diet rich in cholesterol and saturated fat, there is a short-term increase in the levels of lipoproteins [27]. This information is consistent with our results, where the hyperlipidemic diet increased the levels of lipoproteins, and these levels reached over 1,100 mg/dL (**Figure 1**).

The rabbit has a vascular penis as well as the lack of a penile bone are features that make it more similar to the human penis, and therefore a suitable animal model for studying penile structure and erectile dysfunction [10, 11]. These erectile structures in the rabbit penis are also covered by a dense connective tissue, the tunica albuginea (TA), which projects intracavernosal pillars or septa, mainly in the CC [11, 17]. As a result of its anatomical characteristics, the rabbit penis is one of the best models for studies on the effects of hypercholesterolemia on erection [7, 9].

Hypercholesterolemia is one of the most important risk factors in the development of vasculogenic ED. It has been shown that an increase in total cholesterol in men increases the risk of ED [28]. It has also been confirmed in animal studies that hypercholesterolemia and atherosclerotic stenosis in major penile arteries cause ED [29]. Azadzo and Goldstein, [29] noted ED in 16 (76%) of 21 rabbits with experimentally induced hypercholesterolemia and atherosclerosis. However, hypercholesterolemia is a treatable condition; therefore, its effects might be reversible.

ESF and erectile dysfunction

Erection is normally associated with an intracellular cascade of events that change smooth muscle contractility in penile blood vessels and vascular spaces, thereby modifying blood flow and initiating inflation of the CC [13]. However, erection also depends on the collagen and ESF of the underlying connective tissue framework, which exerts passive resistance to the expansion of erectile tissues, thereby creating penile turgidity [13]. Further, ESF provide elastic recoil when the penis returns to a flaccid condition during detumescence [21].

ESF themselves consist of a bundle of fibrillar glycoproteins, such as fibrillins, which are assembled extracellularly and are later embedded with the amorphous elastomeric protein elastin [30]. Thus, the ESF impart viscoelastic properties to tissues and are typically found in structures that, upon application of stretching forces, undergo deformation and then return to the original shape once these forces are removed [30]. Loss or degradation of ESF can cause significant dysfunctions, such as in degenerative and inflammatory disorders [30]. The distribution and structural features of ESF in penile tissues have been investigated in humans [12, 18, 21, 22] as well as in laboratory and food animals [11, 17, 20, 23].

We have previously studied the concentration and distribution of ESF in different regions of the rabbit penis, and the results indicated a close relationship with the known functions of penile tissues [17]. In other study was verified whether ESF content in the CC, corpus spongiosum and tunica albuginea of the rabbit penis undergoes modifications with age [23].

The aim of our study was to determine the content of the ESF of the CC by histochemical and quantitative analysis in rabbits fed with a high cholesterol diet and to determine whether the effect of hypercholesterolemia by long-term was deleterious. In our 24-week study, we observed that hypercholesterolemia affected the volumetric density of ESF.

The long-term effects of hypercholesterolemia and vascular disease due to atherosclerosis in the CC are not well known [9]. Most of the previous studies have been short term, with results suggesting that hypercholesterolemia has reversible effects in cavernosal tissues [7]. In our study, we evaluated the long-term effects and observed a decrease in the ESF in the hypercholesterolemic group (**Figures 2, 4**).

Kim et al. [31] found a decrease in the relaxation of cavernosal smooth muscle cells, endothelial spilling in cavernosal tissues, vacuolization, and an increase in lipid vesicles in smooth muscle cells by electron microscopy and noted that relaxation returned to normal and the other changes to near normal after stopping a cholesterol diet. Nehra et al. [32] reported that a 0.5% cholesterol diet for 16 weeks caused significant decreases in the content of smooth

muscle cells. This decrease in corporeal vascular smooth muscle cells was quantitatively and qualitatively similar to the decrease in men with ED [5]. The findings of Nehra et al. [32] are similar to those of the Karaboga et al. [9], although the duration of exposure was longer in the study of [9]. Thus, changes observed in the penis of hypercholesterolemic animals in the corpora cavernosa suggest a possible association with ED.

Our study attempted to demonstrate the effect of hypercholesterolemia by long-term on the penis of rabbit which has a vascular type similar to humans [3, 8, 9]. In the case of ED it is known that hypercholesterolemia is primarily associated with endothelial changes [3, 5]. Moreover, several factors have been implicated with the onset of ED, including changes in smooth muscle cells, collagen and ESF that form with other elements of the morphological substrate. However, these factors remain scarcely understood. Our study therefore attempted to characterize in a qualitative and quantitative manner, the changes that occur in these elements of the hypercholesterolemic rabbit penis.

One of the characteristics of the ESF is its adaptability in response to a changing environment and different stimuli [30]. The characterization and quantification of the ESF have been shown to be an effective method for the evaluation of morphological and functional changes associated with pathological conditions in humans [12, 18, 20-22] and several animal models [11, 17, 23].

In the specific case of the penis, a change in any one of its components can affect the response of the erectile tissue [13]. The collagen and ESF are the two main structures of the erectile tissue of the penis to allow the increase in circumference and length during tumescence while providing adequate recovery to return quickly to the flaccid state during detumescence [18]. Thus, tissues that are constantly under pressure to stretch are rich in ESF [30] and loss of ESF architecture and function is a pathological feature of a number of degenerative and inflammatory diseases [30]. Despite the importance of ESF in the penis, there are few studies that have accurately characterized this component of ECM and its possible alteration in hypercholesterolemia by log-

term. In young adult rabbits the volumetric density of ESF in the CC is approximately 15% [23], while values for humans are 9% [12, 21] and for young adult rats are 5% [20]. These data suggests that ESF play a particularly important functional role in the rabbit penis. In our hypercholesterolemic rabbits there was a significant decrease of approximately 38.6% in the CC. Observations of the fibrous elements of the extracellular matrix show that while these elements decrease in the CC which suggests a specific behavior in the various regions analyzed.

In conclusion, our results show that hypercholesterolemia by long-term experimentally induced by chicken egg yolk, causes profound changes in the ESF of the rabbit penis suggesting that a possible association with ED often occurs in these patients. We suggest hypercholesterolemia treatment to prevent ED.

Disclosure of conflict of interest

None.

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