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EFFECTS OF ALCOHOL ON HUMAN SPERMATOZOA IN VITRO: SPERM CHROMATIN DISPERSION TEST AND ROS

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Abstract: **This study was carried out to find the detrimental effect on human spermatozoa when exposed to alcohol/ethanol in vitro. For this study semen samples obtained from 12 healthy individuals were washed using the sperm preparation technique and each washed samples were divided into equal aliquots. Then ethanol at various concentrations of 60 mm and 120 mm were added to the aliquots labeled as test samples and samples without ethanol were considered as control samples. All samples with or without ethanol were incubated for 1 hour (60 minutes) at 37^0C. These samples when subjected to semen analysis after exposure to ethanol, it was found that samples added with 60 mm ethanol showed a decrease in motility and vitality, sperms in samples with 120 mm ethanol were almost static with minimum motility. Sperms in both test samples revealed degraded vitality. DNA fragmentation test or SCD when carried out it was revealed that ethanol caused DNA fragmentation in sperms and as the concentration of ethanol increased, DNA fragmentation also increased, motility of sperms decreased and morphology was affected to some extent such as the head and tail defects which was less in the control samples. The sample aliquots with 120 mm ethanol showed morphological abnormalities. The ROS test performed using Agarose-N-gel tubes on the unwashed/raw samples exposed to ethanol in different concentrations revealed the highest oxidative stress displaying dark purple color and unexposed samples showed light pink color indicating low oxidative stress. Reactive oxygen species can damage DNA bases and can cause lesions that block the progression of replication. This study proved that, alcohol when consumed can destroy sperm mobility, vitality and causes high fragmentation levels.**

Keywords: **Ethanol, sperms, DNA fragmentation, motility, vitality, ROS.**

1. INTRODUCTION

Alcohol is a drug which is classified as depressant, when consumed in low/controlled volume it can induce the stimulant effect, but if an individual consumes excess alcohol then he experiences the depressant effect. Alcohol is produced by fermentation of yeast, sugars, and starches. Alcohol gets absorbed rapidly from the stomach and small intestine into the bloodstream. Once alcohol enters the stomach, up to 20% of it gets absorbed and directly enters the bloodstream. Within minutes, alcohol reaches to the brain. Even various nutrients are not capable to pull this off. The remaining alcohol enters into the intestines and is absorbed along with the nutrients. Only a small amount of alcohol is excreted from the body through urine, sweat etc which is not enough to get rid of alcohol in the blood stream. When the alcohol is consumed, the human body is hardwired to metabolize alcohol immediately that means the body stops metabolizing all other things just to metabolize alcohol first because protein, carbohydrates, and fat, can be stored whereas alcohol cannot be stored in the body so it has to be metabolized first and excreted as soon as possible. Alcohol can affect every organ in the consumer's body and can damage the developing fetus in woman who is pregnant. Also alcohol has the potential to severely damage sperms. If alcohol consumption is fairly regular then it will affect sperm motility and vitality in men. Heavy alcohol consumption lowers the sperm count leaving the person infertile and the levels of free radicals generated due to alcohol can break DNA strands in the sperm cells. The aim of this study was focused on the effects of alcohol on sperms and mainly on the level of DNA fragmentation and oxidative stress caused in sperm samples due to alcohol in vitro.

2. MATERIALS AND METHODS

For the study of alcohol effects on sperms in vitro, Semen samples from 12 healthy men (non-alcoholic) aged 21 – 27 were collected in wide mouthed sterile collection containers and Sperm samples were obtained during 3-4 days of sexual abstinence and liquefied for 20-30 minutes at room temperature. The quality of the sperm is judged on the basis of its morphology and movement patterns or motility and vitality. For in vitro study, sperm samples from healthy individuals were washed by swim up from pellet method, which is a very common method for retrieval of viable sperms and has a high success rate. All the washed samples were divided in 3 equal aliquots of 0.5ml. Ethanol in different concentrations of 60mm and 120 mm respectively were added to 2 sample aliquots labeled as test samples and 1 aliquot was un-exposed to alcohol labeled as control sample. These samples were incubated for 60 minutes at 37^0C and subjected to semen analysis according to the guidelines set by world health organization. Sperm count and motility evaluation was done under bright field microscope, morphology was studied by making smears of sperms on the slides and was stained using Giemsa stain. pH was observed by using a pH strip and vitality of the sperms were noted by staining sperms with eosin and nigrosin stain. Morphology was judged by referring the types of sperm deformities proposed by WHO (World Health Organization., 2010). Motility was calculated by using a formula: 100 X (number of motile spermatozoa)/ (total number of spermatozoa counted). Also for calculating vitality percentage: 100 X (number of viable spermatozoa)/ (total number of spermatozoa counted). More than 500 spermatozoa per ejaculate were evaluated for estimation of sperm motility and vitality. The outcome of this analysis was noted. The rest of the control and test samples were used for DNA fragmentation test.

Sperm Chromatin Dispersion Test:

The test aliquots were added with various concentrations of alcohol (60mm and 120mm respectively) to see what concentration of alcohol will affect the DNA of the sperms. The control sample (0.5 ml) with no ethanol and 2 test samples (0.5 ml each) mixed with ethanol incubated at 37^0 C for 60 minutes were subjected to DNA fragmentation test. Requirements per sample were 3 coated slides, 1 for control and other 2 for test. The procedure involved addition of sperm samples of both test and control to agarose tubes which were previously boiled for 2 minutes. After adding and mixing the 40 µl droplets of sperms from both control and test samples in the agarose tubes, a total of 150 µl droplets of sperm samples from each tube was extracted using micropipette and was laid on pre-coated slides. Cover slips were placed on the droplets for even distribution of sperm sample immersed in agarose matix on the slide. The 3 slides of 1 control and 2 test samples were kept inside a fridge for 6 minutes to solidify the agarose gel in which sperms were added. After this step, cover slips were removed in such a way that the gel integrity was not disturbed. Acid denaturant (1 ml) was laid on the slide for 7 minutes and was drained. Then 1ml of lysis solution was overlaid on the slide for 20 minutes and was drained. Then both test and control slides were washed with 20 ml of distilled water and were overlaid with 1 ml each of dehydrating solutions 1, 2, 3 for 2 minutes each. After disposing or draining the dehydrating solutions, the slides were air dried for few minutes. Stain was prepared by mixing stain solution and stain diluting solution. The slides prepared for each of the 12 samples were stained for 3 minutes and were washed in a beaker filled with tap water to remove excess stain. Slides were again air dried for few minutes before observing under bright field microscope for halos. For calculating percentage of DNA fragmentation: 100 X (Number of spermatozoa with fragmented DNA)/ (Total number of spermatozoa counted). More than 450 spermatozoa per ejaculate were evaluated for estimation of sperm DNA fragmentation (Omkar pokharkar et al., 2015). For this study, A Sperm DNA Fragmentation Detection Kit called 'CANfrag' [REF No.CA-001], provided by 'CANdORE Bioscience', Ahmedabad, Gujarat was used to assess the levels of sperm DNA fragmentation in the samples. This entire procedure was followed for all 12 samples and it took around 18 days (10^{th} February –27^{th} February, 2015) for completion of the study and to arrive at results.

ROS (reactive oxygen species) test:

A Reactive Oxygen Species detection kit called 'CANros' [REF NO.CA-002] also provided by 'CANdORE Bioscience' was employed for mapping oxidative stress in exposed and un-exposed samples. Along with washed samples of test and control, 2 aliquot (test) of raw samples of 0.5 ml each was also added with ethanol in various concentration of 60mm and 120 mm respectively and control raw sample of 0.5ml was un-exposed to alcohol. After incubation of both control and test samples for 60 minutes in an incubator at 37^0C, ROS test was conducted by heating the Agarose N-Gel tubes in boiling water at 90-100ºC for 2 minutes to melt the gel. The tubes were cooled down for 2 minutes at room temperature before addition of the sample. 0.2 ml of semen sample was added and mixed thoroughly with the melted agarose gel and air bubbles were avoided. The Agarose-N-gel tubes were then placed in an incubator at 37ºC for 60 minutes. After incubation the color changes were observed immediately and were compared with the color code mentioned in the ROS kit to determine the level of oxidative stress present in the sample (Omkar pokharkar et al., 2015). The results of this test were noted.

3. RESULTS

The pH of both exposed samples (60mm and 120 mm) was in the abnormal range. Alcohol affected the pH of the samples in which it was added and increased with the increase in the concentration of alcohol. Morphology of sperms was affected by alcohol to some extent only in samples with 120 mm ethanol/ 0.5 ml of sperm sample. The sperm count in all the samples remained in normal range at these concentrations. The average sperm count for all samples throughout the study was in the range of 60 to 70 million sperms/per ml. The average percentages of motility and vitality obtained for all 12 samples were calculated and framed in the table below.

Results are mentioned in the chart and table form below:

Table I. Semen parameters for both alcohol exposed and un-exposed samples

SEMEN PARAMETERS OF BOTH EXPOSED AND UN-EXPOSED SAMPLES TO ALCOHOL IN VARIOUS CONCENTRATIONS:					
	Motility (%)	Vitality (%)		morphology	pH
Un-Exposed samples	75.21%	72.12 %		Overall normal	7.2
Exposed samples (60 mm)	60.24 %	57.25 %		Overall normal	8.3
Exposed samples (120 mm)	45.19 %	44.36 %		Slight abnormality	8.5

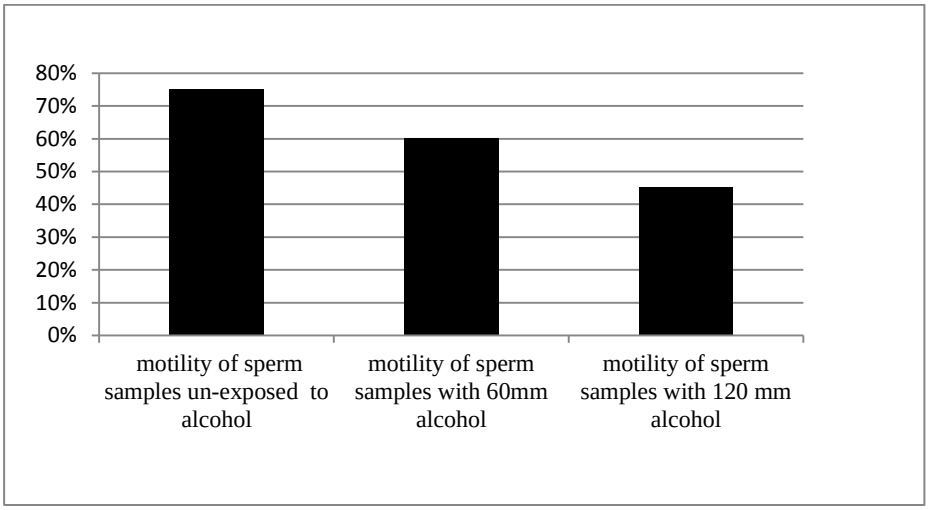

Chart I. Effect of alcohol on sperm motility

The motility in sperm samples was decreased due to alcohol exposure. Motility in samples un-exposed to alcohol showed excellent motility and samples exposed to the alcohol showed a decrease in the motility as the concentration of alcohol increased.

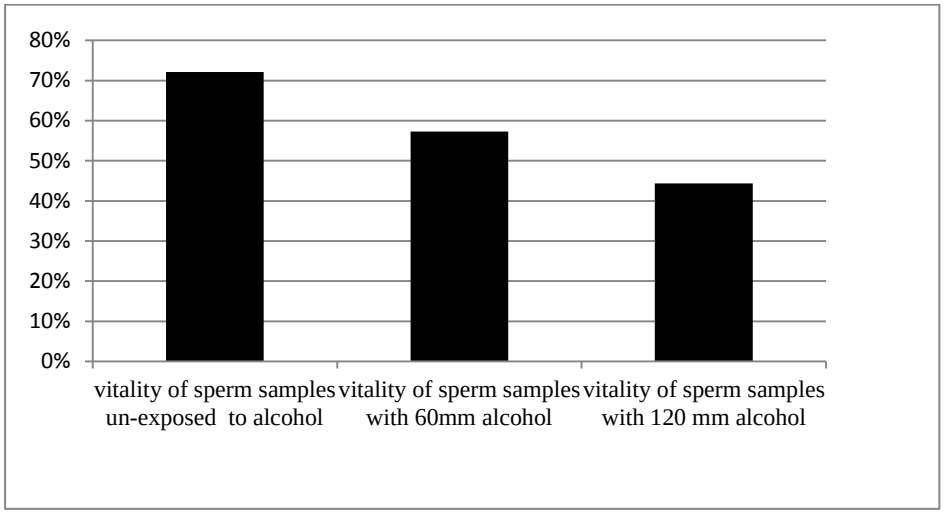

Chart II. Effect of alcohol on sperm vitality

The chart mentioned above indicates that, the sperm un-exposed to the alcohol showed an excellent range of vitality percentage while the exposed samples at various concentration of alcohol showed a constant drop in the vitality percentage. The table mentioned below represents the average DNA fragmentation percentages obtained for all 12 samples.

Table II. DNA fragmentation in exposed and un-exposed samples

SPERM DNA FRAGMENTATION IN BOTH EXPOSED AND UN-EXPOSED SAMPLES TO ALCOHOL.	
Un-exposed samples	8.24 %
Exposed samples (60 mm)	28.37 %
Exposed samples (120 mm)	39.43 %

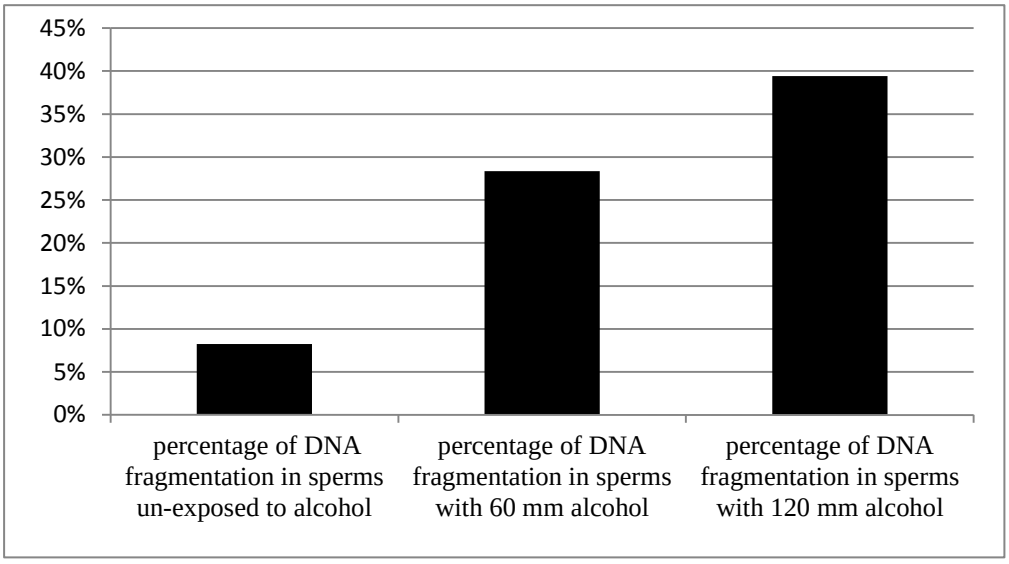

Chart III. Sperm DNA fragmentation due to alcohol

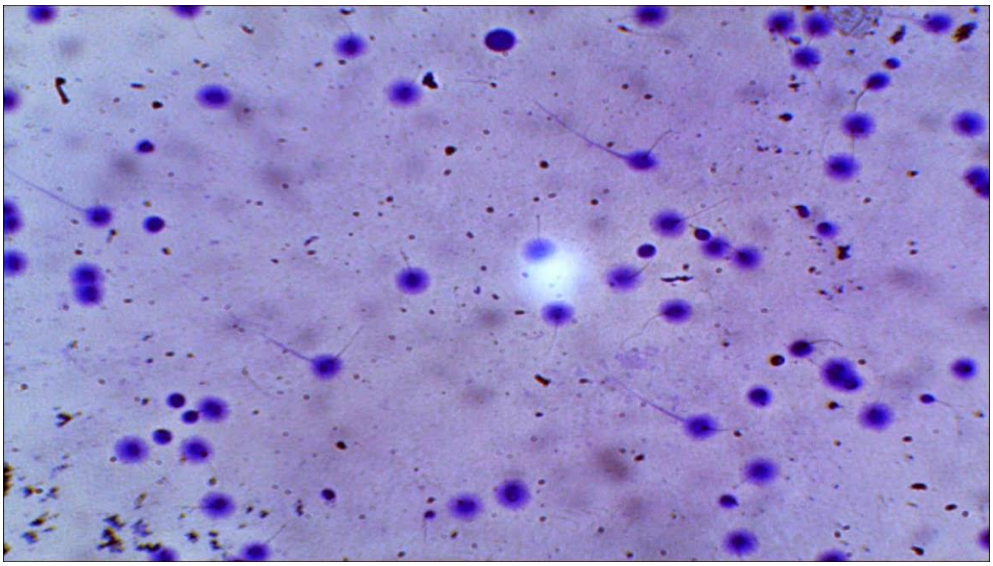

Figure I. Original picture of DNA fragmentation slide un-exposed to alcohol (control slide)

It is evident from the picture above that, the samples un-exposed to ethanol showed maximum sperms with intact DNA displaying large halos. On the other hand, the samples exposed to ethanol in concentrations of 60mm and 120mm showed maximum sperms with fragmented and degraded DNA and revealed minimum sperms with intact DNA displaying small and no halos. Below is the picture showing fragmented and degraded sperms.

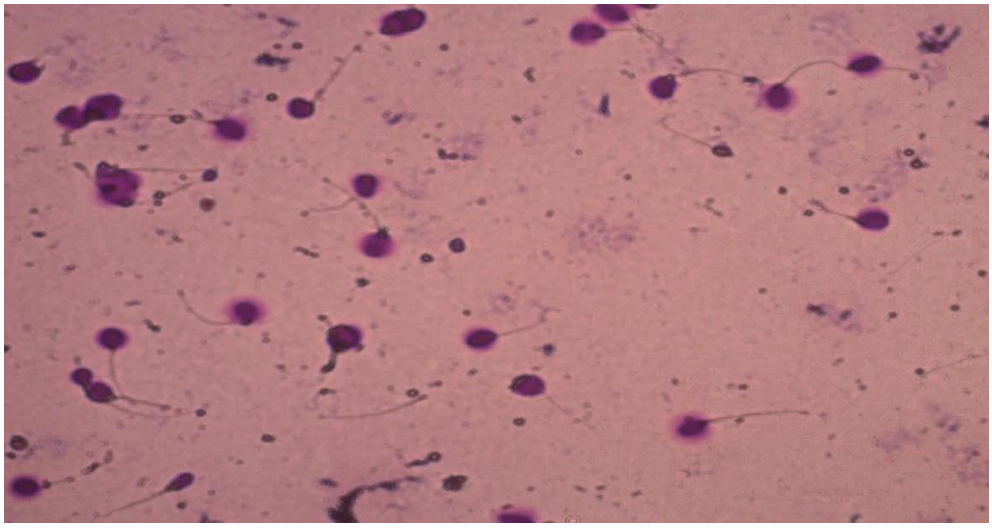

Figure II. Original picture of DNA fragmentation slide exposed to alcohol [60mm and 120mm (test slide)]

ROS (reactive oxygen species) TEST.

ROS test were performed using freshly collected semen samples. Test aliquot of core samples exposed to alcohol at concentration of 60mm/0.5 ml and the 2nd test aliquot exposed to alcohol at concentration of 120mm/0.5 ml of semen sample produced dark purple colour. On the other hand control aliquot un-exposed to alcohol showed light pink colour.

Table III. Reactive oxygen species result for alcohol

Reactive oxygen species(ROS)	color	Color code
Highest levels of ROS in samples (60 mm and 120 mm).	Dark purple	
Low levels of ROS (un-exposed samples).	light pink	

Dark purple color was obtained for both test samples which indicated that highest level of oxidative stress was induced in the sample due to alcohol. Whereas samples un-exposed (control) to alcohol showed light pink color. Alcohol, at even low concentrations can achieve highest levels of ROS.

4. CONCLUSION

The study revealed that alcohol in high concentrations can damage the sperms in every possible way and make it non-viable. The results mentioned above point out to the fact that as the alcohol/ethanol concentration increases, the sperm quality decreases. Motility in control samples was greater as compared to both the test samples (see Chart I). Vitality in sperm samples went on degrading as the ethanol concentration increased (see Chart II). Morphological abnormalities were mostly scored on the test slides with 120 mm ethanol and samples with 60 mm ethanol and control samples with no ethanol showed a normal range of morphological defects and pH of samples also increased with the increase in concentration of alcohol (see Table I). This indicated that the alcohol in high concentrations can damage the physical appearance of sperms. The number of sperms with fragmented DNA was most prominent in the test samples with 120 mm ethanol and 60 mm ethanol. On the other hand, control samples showed fewer fragmentations (see Table II and Chart III). The sperms with fragmented DNA disperse small halos and most of the time no halos were observed (see Figure II) and sperms with intact DNA disperse big halos (see Figure I). By this method, it becomes easy to map the fragmented and non-fragmented DNA of the sperms. ROS test was conducted to evaluate the findings and it gave the further evidence of fragmentation caused due to alcohol in vitro by achieving dark purple color in the Agarose-N-gel tubes by reduction of nitro blue tetrazolium and un-exposed samples managed to display light pink color indicating low levels of free radicals (see Table III). Reactive oxygen species has the potential to damage DNA bases and can cause lesions that blocks progression of replication. Damaged sperm chromatin contains base adducts and the prominent adducts found in human sperm DNA are 8OHdG (8-hydroxy-2'-deoxyguanosine or 8-oxo-7,8-dihydro-2'–deoxyguanosine) present in nuclear and mitochondrial DNA. Ethenonucleosides such as 1,N6-ethenoadenosine and 1,N6-ethanoguanosine are found in sperm DNA. Single strand breaks are the direct consequences of oxidative attacks on sperm DNA.

5. DISCUSSION

As the coin has 2 sides, alcohol too have a good and a bad side. It depends how the individual use the alcohol and for what purpose. Alcohol is in use for scientific purposes as well as for production of beverages such as beer and wine for centuries. Applications of Alcohol in the field of science date back to 18th century when it was used for dressing up the wounds and to sterilize the equipment for surgery (civil war in U.S. 1850's). Today along with these applications, alcohol is also used as antifreeze, preservatives, fuels, and also as solvents. But high consumption of alcohol is not beneficial in any way; Addiction to alcohol could severely damage the reproductive system of that individual making him infertile. A few studies around the world indicated that consumption of alcohol every day can effectively reduce the sperm count and quality of the sperm degrades as the consumption of alcohol increases. The people with disorders such as 'auto brewery syndrome' (gut fermentation syndrome) have very less reproductive capabilities. In this type of syndrome body produces alcohol through endogenous fermentations within the digestive system. A fungal infection of 'Saccharomyces cerevisiae' in gastrointestinal tract is responsible for this condition. This constant production of alcohol leads to several other body problems such as kidney failure, liver dysfunction and leydig cells in the testes are damaged which are responsible for production of testosterone and the levels of testosterone in blood drops and also Sertoli cells present in the testes are affected which interferes with sperm maturation. Immature sperms present in the ejaculate are not capable to move and fertilize the egg. The aim of this study was focused on in vitro effects of ethanol which involved direct exposure of ethanol to sperms which caused more damaging effect. However, in vivo study of effects of alcohol on sperms would produce more promising and accurate results as compared to in vitro study because alcohol undergoes elimination by various metabolic mechanisms that occur in the body. The enzymes involved are aldehyde dehydrogenase, alcohol dehydrogenase, cytochrome P450 and catalase. The interaction occurring between alcohol byproducts obtained after metabolism and other cell components, leads to the formation of dangerous compounds such as reactive oxygen species (ROS). Alcohol metabolism occurs primarily in the liver. Alcohol undergoes detoxification procedure and gets eliminated from the blood by the process termed as oxidation. Oxidation inhibits accumulation of alcohol and prevents destruction of cells and other organs in the body to some extent. So the in vivo results would be much different than in vitro study as the alcohol undergoes metabolic paths. In in vitro study direct exposure of ethanol caused high fragmentations and more damage was inflicted to the sperm parameters (motility, vitality, morphology, and pH). It can be concluded that, consumption of alcohol is associated with degradation of sperm quality which can be reversed to some extent upon alcohol consumption discontinuation which would reduce the blood alcohol concentration (BAC) and intake of antioxidants on a regular basis would maintain low concentration of free radicals and will protect the DNA of sperms from damage.

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