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The effects of male aging on semen quality, sperm DNA fragmentation and chromosomal abnormalities in an infertile population

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Abstract
Purpose To investigate the effects of male aging on semen quality, DNA fragmentation and chromosomal abnormalities in the spermatozoa of infertile patients and fertile men.
Methods Semen samples of 140 infertile patients (24–76 years) and 50 men with proven fertility (25–65 years) were analyzed according to WHO guidelines. DNA fragmentation was detected by TUNEL assay, while aneuploidy was assessed by FISH.
Results In the patient group, semen volume and vitality of spermatozoa decreased significantly with age, while sperm concentration showed a statistically significant increase with age. DNA fragmentation as well as disomy of sex chromosomes and disomy 8 did not show a statistically significant change with age. However, the diploidy rate was significantly increased with patient's age. In the control group, conventional semen parameters as well as DNA fragmentation and chromosomal abnormalities did not show a statistically significant with age.
Conclusion Increased age in infertile men is associated with an increase in sperm concentration and diploidy, as well as a decline in semen volume and sperm vitality. However motility, morphology and DNA fragmentation are not affected by male age.

Keywords Aneuploidy · DNA fragmentation · Male infertility · Paternal age · Semen parameters

Capsule Increased age in infertile men is associated with an increase in sperm concentration and diploidy, as well as a decline in semen volume and sperm vitality.

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Introduction

While female fertility ends at the entrance into menopause around the age of 50, men generally do not experience an unavoidable and clear-cut cessation of reproductive capacity. The effects of male aging on semen quality, DNA fragmentation and chromosomal abnormalities in infertile patients and fertile donors were reported since 1970 but the results are conflicting.

Conventional semen analysis, including ejaculate volume, sperm concentration, motility, and morphology determined according to World Health Organisation (WHO) criteria, are the first step in the evaluation of male infertility. Among the basic semen parameters studied, semen volume, percentage of motile spermatozoa, and the percentage of normal morphology were more consistently reported to decrease with age. However, no consistent data confirm that sperm concentration also decline with advancing years [1, 2]. Semen analysis provides limited prognostic information on fertility, and recent studies indicated that the integrity of sperm DNA might be a more accurate and precise predictor of fertility [3] an intact DNA is necessary for the correct transmission of genetic materiel to the next generation. High levels of sperm DNA damage have been reported to affect fertility potential, increase the risk of recurrent miscarriages, decrease the chances of a successful implantation, and possibly lead to negative effects on the health of offspring [4, 5]. Sperm DNA integrity as assessed by terminal desoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) [6], sperm chromatin structure assay (SCSA) [7, 8], and Comet assay [9], were also shown to be compromised with advancing age. However, this notion was not supported by all studies [10–12], and the results varied according the technique used for the detection of DNA damage.

An important issue in the evaluation of age related alteration of male fertility is the frequency of hereditary disease in offspring. Whereas it is well established that women >35 years of age bear a higher risk of conceiving genetically abnormal offsprings [13, 14], a correlation with paternal age is still at issue. For instance, Sloter et al., have shown that advancing male age is associated with a gradual and significant increase in the risk of fathering children with various chromosomal defects on a total of 320 unselected patients consulting their IVF and Urological Center [15]. In contrast, Luetjens et al. [16] did not find a significantly higher risk of producing chromosomally abnormal offsprings for men of advanced age. These conflicting results can be explained by the heterogeneity of the selection subjects, the age groups and the methods of analysis. The study settings and populations are heterogeneous, with more than half based on infertility clinic or assisted conception populations, while the others used volunteers recruited from advertisements or sperm banks. In addition, men at older ages (e.g. >50 years) were underrepresented in many of these clinical studies, which limited statistical power and prevented the determination of the shape of the relationship between age and semen quality and sperm DNA damage. Moreover, only few studies were controlled for abstinence time.

In order to give more accurate results, we have designed a prospective study to investigate the effects of male ageing on semen quality, DNA fragmentation and chromosomal abnormalities in the spermatozoa of infertile patients and fertile donors. Semen samples were controlled for abstinence time and older men are included in the present study.

Materials and methods

Patients

A total of 140 men consulting for infertility evaluation at our laboratory of Cytogenetic and Reproductive Biology, Farhat Hached University Teaching Hospital, Sousse (Tunisia) were included in this study. Furthermore, 50 healthy donors, with proven fertility, were also included in the study in order to evaluate possible differences between fertile and infertile men. All the patients and donors had no history of chemotherapy, radiotherapy, or chronic illness.

This protocol was approved by the local ethics committee and all patients and controls had previously given informed consent for the study.

Semen analysis

Semen samples were collected by masturbation in to sterile cups following 3 days of sexual abstinence and semen analysis was performed after semen liquefaction for 30 min at room temperature. Basic semen parameters (volume, vitality, concentration, and total motility) were assessed according to the World Health Organization guidelines [17]. Sperm concentration was determined with an improved Neubauer Hemacytometer® counting chamber. Sperm morphology was evaluated using the Diff-Quick staining method. At least 100 spermatozoa per patient were examined at a magnificent of X 100 according to the David classification [18].

Semen preparation for FISH analysis and TUNEL assay

An aliquot of the fresh semen was washed twice in Phosphate Buffered Saline (PBS, pH 7.4) and centrifuged at 400 g for 5 min. The sediment was then fixed in methanol/acetic acid (3: 1) for at least 30 min at 4°C. The fixed specimens were smeared on slides and stored at –20°C until further processing.

Measurement of DNA fragmentation

The presence of apoptosis-related DNA strand breaks in spermatozoa was evaluated by the terminal desoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick-end labeling (TUNEL) assay, using the ApopTag® Apoptosis Detection Kits (QBiogene, Paris, France) in controls and patients. For cell permeabilisation, slides were incubated in phosphate buffer saline (PBS) with a solution of 1% Triton X100 (Sigma). The procedure was carried out according to the manufacturer's instructions. Briefly, the specimens were washed twice in PBS 1X, equilibrated with the equilibration buffer at room temperature for 10 s and incubated in a dark moist chamber at 37°C, for 1 h, with the Terminal Desoxynucleotidyl Transferase (TdT) solution in order to allow DNA elongation. After stopping the enzyme reaction, the slides were washed twice in PBS and the DNA elongation was revealed by incubation of the cells with anti-digoxigenin antibody coupled to peroxidase, during 30 min in a dark moist chamber. The peroxidase was revealed with DiAminoBenzidine (DAB). Slides were then counterstained with Harris' haematoxylin (RAL, Martillac, France) and finally mounted using Faramount mounting (Dako, Carpinteria, CA, USA).

Controls were also included in every experiment: for the negative control TdT was omitted and we have include proteinase K digestion to control for nonspecific incorporation of nucleotides or for nonspecific binding of enzyme-conjugate, whereas positive controls were generated by incubating the sperm cells for 10 min at room temperature with DNase I.

Slides were observed under a microscope (Zeiss, Oberkochen, Germany) equipped with a 100 magnification lens. Spermatozoa with fragmented DNA had brown-

colored nuclei, whereas the other cells were blue-gray (counter coloration with Harris's haematoxylin). On each slide, approximately 500 cells were counted, and the percentage of spermatozoa with fragmented DNA (DFI) was calculated.

Aneuploidy analysis

Fluorescence in situ hybridization, which employs sequence-specific DNA probes incorporated with fluorescently labeled nucleotides, was carried out on each patient and control, using alpha centromeric probes for chromosomes 8, X and Y.

Sperm head decondensation

In order to render the sperm chromatin accessible to DNA probes, slides were incubated in NaOH 1N, at room temperature for 2 min. The slides were distilled water washed. Then, they were dehydrated through an ethanol series (70–90–100%) and air-dried.

DNA probes

The probe mixture for triple FISH consisted of a repetitive DNA sequence of centromeric probes for chromosome X (pDMX1) labeled fluorescein isothiocyanate (FITC), for chromosome Y (pLAY5.5) labeled Rhodamine and for chromosome 8 (pZ8.4) labeled FITC and Rhodamine. The probes were provided by the University of Bari (Bari, Italy).

The use of an autosomal probe, in addition to X and Y probes, allowed the distinction between disomy and diploidy.

Hybridization procedure

Slides were incubated in a denaturation solution of 70% formamide, 20X standard saline citrate (SSC) (pH 5,3) and distilled water at 72°C for 2 min. Slides were snap-cooled in 70% ethanol at –20°C for 2 min and then dehydrated through an ethanol series (90–100%) at room temperature. The probes, precipitated and denatured at 72°C for 8 min, were applied directly to the slides which were then covered with a cover slip and sealed with rubber cement. Slides were hybridized for 2 h in a dark humidified container at 37°C. Finally slides were washed in 1× SSC, counterstained with 4′,6-diamidino-2-pheneylindole (DAPI) and stored in the dark at 4°C prior to carrying out microscopic observation.

Scoring criteria

The slides were observed using an Axioplan epifluorescence microscope (Leica, Wetzlar, Germany) with the appropriate set of filters: single band DAPI, FITC, and Rhodamine. For each probe a minimum of 500 spermatozoa were counted per patient. Only intact spermatozoa bearing a similar degree of decondensation and clear hybridization signals were scored; disrupted or overlapping spermatozoa were excluded from analysis.

Statistical analysis

Statistical analysis was performed using SPSS.13 (SPSS, Chicago, IL, USA). All variables were initially tested in order to determine variance homogeneity and data normality. Data are represented as Mean±Standard deviation (SD). Group comparisons were made using student's *t*-test. Pearson's correlation was performed to examine the relationship between paternal age and standard semen parameters, DNA fragmentation and aneuploidy rate. All hypothesis testing were two sided with a probability value of 0.05 deemed as significant.

Results

Characteristics of study population

A total of 140 semen samples from infertile patients were analysed regarding the semen parameters, DNA fragmentation, and aneuploidy. Subject's ages ranged from 24 to 76 years. The mean age was 37.65 years.

56.83% of the subjects were between 30 and 40 years old; 7.91 % were <30 years and 35.25 % were >40 years old. The results of our study were allocated to four age groups:

- Younger than 30 years (27.09 years in average; $n=11$)
- 30–39 years (34.61 years in average; $n=79$)
- 40–49 years (42.61 years in average; $n=40$)
- ≥50 years (54.50 years in average; $n=10$)

For control group, subjects ranged in age from 25 to 65 years with a mean of 37.34 years. Also they were distributed into four groups:

- Younger than 30 years (27.12 years in average; $n=10$)
- 30–39 years (35.04 years in average; $n=16$)
- 40-49 years (43.50 years in average; $n=14$)
- ≥50 years (56.50 years in average; $n=10$)

Effects of age on semen parameters

The results of the basic semen parameters according to male age were presented in Table 1. For infertile patients, we show that semen volume, vitality, total motility, and percentage of normal morphology decline with advancing age. Only sperm concentration increases with advancing age. However no statistical significance was demonstrable between any of the

Table 1 Descriptive statistics and comparisons of conventional semen parameters between the four age subgroups in both fertile and infertile men

Age group (Years)	Volume (ml)	Concentration (X 10^6/ml)	Total motility (%)	Abnormal forms (%)	Necrozoospermia (%)
Fertile men					
20–29 (n=10)	2.83±0.92	95.75±22.93	53.75±4.43	58.38±9.56	22.28±05.70
30–39 (n=16)	2.87±0.53	93.07±29.46	51.33±6.81	55.78±7.26	25.06±3.30
40–49 (n=14)	2.48±0.31	100±46.20	48.12±5.12	57.66±7.39	21.36±2.80
50–70 (n=10)	2.06±0.11	99.12±31.34	47.56±3.53	58.12±10.94	24.35±3.67
Infertile men					
20–29 (n=11)	3.26±1.63	62.09±40.92	26.36±17.04	74.18±13.33	32.82±25.90
30–39 (n=79)	3.31±1.58	76.07±59.86	29.00±15.84	79.22±17.26	31.06±19.00
40–49 (n=40)	2.08±1.31	97.97±86.13	25.54±14.29	81.66±17.39	36.76±21.56
50–70 (n=10)	2.46±1.11	98.12±61.34	22.00±17.43	85.12±10.94	52.62±30.38

age groups of the patients (p>0.05). In addition total sperm count did not significantly differ with age group (p>0.05).

Using Pearson's correlation test, we didn't found a statistically significant relationship between age, and percent motility (r=−0.088; p=0.305), percent normal morphology (r=0.026; p=0.765), and total sperm count (r=0.025; p=0.768). However a significantly inverse relationship was shown between patient's age, semen volume (r=−0.183; p=0.032), and vitality (r=−0.219; p=0.01) (Fig. 1). Sperm concentration was significantly and positively associated with male age (r=0.196; p=0.021) (Fig. 2a).

For the controls, semen volume, sperm concentration, total sperm count, morphology and progressive motility did not differ significantly between the four age groups. In addition, these conventional semen parameters did not show a statistically significant correlation with age (p>0.05).

Effects of age on DNA fragmentation

The TUNEL assay results stratified by male age were presented in Table 2.

In infertile patients there was a trend for increased levels of DNA fragmentation with advancing age but these results were not statistically significant (p>0.05). No correlation was found between male age and level of DNA fragmentation (r=0.08; p>0.05).

In the controls there was no statistically significant difference between the four age groups in the percentages of cells with fragmented DNA. In addition using the Pearson rank correlation coefficient we did not find a significantly correlation (r=−0.094; p=0.516).

Effects of age on aneuploidy rate

The results of aneuploidy frequencies for chromosomes 8, X, and Y stratified by male age are also presented in Table 2. Only sperm diploidy was significantly different among patients groups, with an obvious increase with advancing age (0.38 % in men younger than 30 years versus 1.02 % in patients 50 years and older; p<0.01).

A Pearson correlation coefficient was employed in order to study the relationship between the patient's age and the various

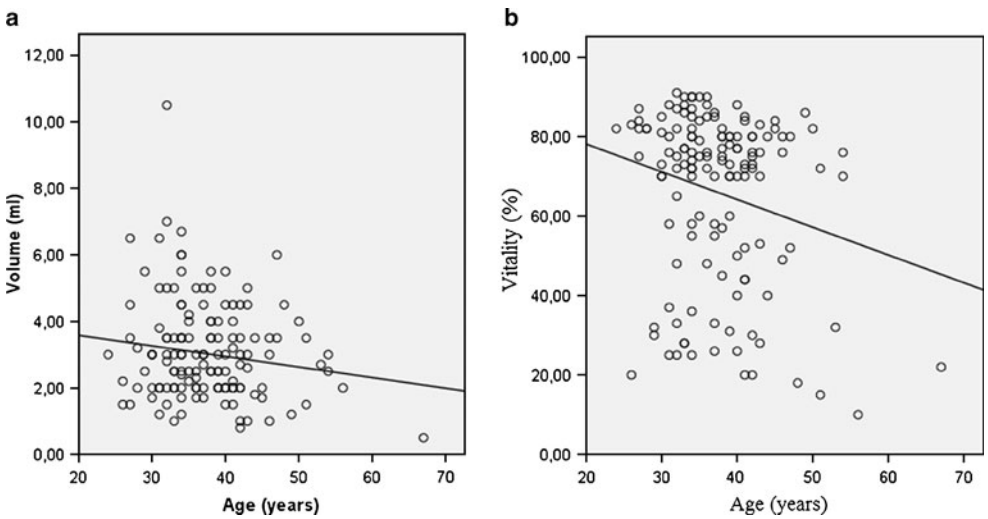

Fig. 1 Scatter graph illustrating the negative associations between age and semen volume (**a**; r=−0.183; p=0.032) as well as age and vitality of spermatozoa (**b**; r=−0.219; p=0.01)

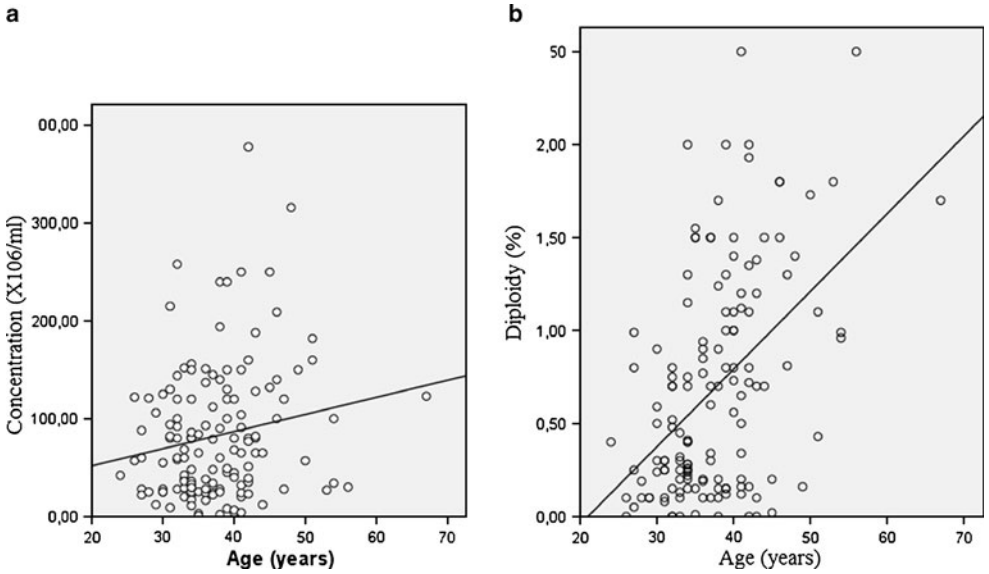

Fig. 2 Scatter graph illustrating the positive associations between age and sperm concentration (**a**; r=0.196; p=0.021) as well as age and diploidy rate (**b;** r=0.544; p=0.01)

abnormality frequencies. There was no correlation of male age with the disomy for chromosomes 8, X, Y, and XY (p>0.05). However, the diploidy rate was significantly increased with patient's age (r=0.544; p=0.01). This association between age and diploidy was presented in Fig. 2b.

For fertile men, there was no correlation of donor age with the frequency of X- or Y- bearing sperm, disomy for chromosomes 8, X, Y, XY, or diploidy (p>0.05).

Discussion

Many studies have analysed age-dependant variations of sperm parameters but only few were controlled for abstinence time. In addition, the selection of subjects, the age groups (rarely include males >60 years), and the methods of analysis are heterogeneous and the results are conflicting. The particularity of the present study is that semen samples were controlled for abstinence time (after 3 days of abstinence) and older men were also included in this study. Our results found that increased age of infertile men is associated with a decrease in semen volume, a decrease in sperm vitality, and an increase in sperm concentration. Sperm motility and percent normal sperm morphology decrease with age but this decline was not statistically significant. Classical parameters of spermiograms did not change significantly in over 800 fertile men between 21 and 50 years of age. Unfortunately, elderly men were not included in that particular study [19]. However Levitas et al. detected a statistically significant and inverse relationship between semen volume, sperm quality, and patient age, but the patients had a longer period of sexual abstinence before the testing [20]. So we show that potential confounders that might explain changes with age, such as duration of abstinence and age groups, could change result totally. The methodologically superior studies

Table 2 Descriptive statistics and comparisons of DNA fragmentation and chromosomal abnormalities between the four age subgroups in both fertile and infertile men

Age group	DFI (%)	Disomy X (%)	Disomy Y (%)	Disomy XY (%)	Disomy 8 (%)	Diploidy (%)
Fertile men						
20–29 (n=10)	10.50±1.41	0.10±0.01	0.33±0.11	0.48±0.03	0.13±0.05	0.09±0.06
30–39 (n=16)	10.37±4.39	0.15±0.03	0.39±0.12	0.54±0.19	0.16±0.06	0.14±0.09
40–49 (n=14)	9.93±4.07	0.17±0.04	0.35±0.10	0.52±0.16	0.17±0.10	0.12±0.06
50–70 (n=10)	9.00±5.65	0.14±0.06	0.46±0.15	0.55±0.21	0.14±0.15	0.13±0.04
Infertile men						
20–29 (n=11)	26.23±13.49	0.36±0.30	1.08±0.59	1.62±1.02	0.47±0.34	0.38±0.37
30–39 (n=79)	27.66±15.84	0.38±0.31	1.09±0.75	1.75±1.07	0.69±0.83	0.44±0.51
40–49 (n=40)	30.42±16.85	0.44±0.30	1.37±0.85	1.96±1.16	0.68±0.52	0.61±0.58
50–70 (n=10)	31.62±18.01	0.58±0.18	1.22±0.31	1.98±0.55	0.61±0.16	1.02±0.53

suggest that in general the semen volume [21–23], the percentage of motile sperm cells [21, 23] and the percentage of the sperm cells with normal morphology [23–25] decline with age. However, no consistent data confirm that sperm concentration also decline with advancing years. Abstinence adjusted studies do not provide a uniform picture. A significant age–dependent decrease [2, 25] as well as constant values over the age range [19] or even a non significant age dependent increase with age [26] have been detected in healthy men. In infertile patient's sperm concentration remains unalterated [22] or increases [21, 23, 26–29]. In our study we had found that sperm concentration increases with age in infertile men but remains constant in fertile men. For total sperm count we show that it did not significantly differ with age group of fertile and infertile men. Few studies have analyzed the total sperm count and this decreases with age in fertile men [2] but remains constant in infertile men [21].

Age may not only impact semen quality, but also the genetic integrity of the sperm. Sperm DNA damage has been attributed to a variety of intra- and extra-testicular factors. Probably the most important are the improper packaging and ligation during sperm maturation [30, 31], the production of reactive oxygen species by oxidative stress [32, 33] and the defective apoptosis before ejaculation [33, 34]. However the impact of age on sperm DNA fragmentation is still an unsolved question. Several techniques are currently available that assess sperm DNA damage directly or indirectly by evaluating sperm chromatin compaction. The most common tests are SCSA, COMET, and TUNEL assays [31]. Using the TUNEL assay, we did not found a significant association between the paternal age and the levels of DNA fragmentation in infertile and fertile men. Using the same technique, our findings are in accordance with the data of Colin et al. [10] and in discordance with the results of Vagnini et al. [6] and Plastira et al. [29] who reported that male aging affects the chromatin integrity of spermatozoa, but only in infertile population. Using a different assay for measuring DNA strand damage in sperm, the SCSA which measures the susceptibility of sperm DNA to in situ acid induced denaturation, Spano et al. [7] found a strong association of DNA fragmentation index with age among men 18–55 years olds. Similar trends were found by others investigators [8, 35–38]. In contrast, Schmid et al. [11] using the same methods, reported that male age only influences single strand breaks and age was not associated with sperm DNA damage under neutral conditions, which is thought to represent double strand DNA breaks. Using a modified Nicoletti assay, Winkle et al. [12] suggest that the amount of spermatozoa with fragmented DNA is not affected by male age. So we show that the results of literature varied according the technique used, but the majority of studies show a positive relationship between age and DNA damage. The limited sample size analyzed in our study, compared to other studies relating paternal age and sperm DNA damage, may explain the lack of significant association of DNA fragmentation and male age.

Initial attempts to relate paternal age and chromosomes abnormalities were only carried out when the interspecies human/hamster fertilization system became available. The results were controversial in part because there were few analyzable metaphases, and also because the oldest men studies were just 44 years of age. With the advent of FISH, it became possible to analyze a much larger number of sperm. Studies which analyzed the age–dependent alteration of aneuploidy frequency in chromosomes are severely limited due to low case numbers. So far no age effect has been found for aneuploidies in chromosomes 6, 8, 12, 13, 14 or 18 [16] and varying results for chromosomes 1, 9, 21, X, and Y. Using a triple color FISH X-Y-8, we did not find a paternal age effect on sex chromosomes disomy (XX, YY, XY) in infertile patients and in donors. Our results are in accordance with the data of Bosch et al. [39, 40] and Luetjens et al. [16], but in discordance with others studies which found an effect of age on the production of disomic sex chromosomes with varying results for XX, YY, XY [41–47].

A statistically significant tendency to a linear increase of diploidy with age was observed for our infertile men. The same result was shown by Bosch et al. [39, 40]. However Martin et al. did not support this correlation between paternal age and diploidy [43].

In conclusion our study demonstrated that increased age in infertile men is associated with an increase in sperm concentration and diploidy rate, as well as a decline in semen volume, and sperm vitality. Moreover we had found that sperm DNA fragmentation as well as disomy X, Y, XY, and 8 were not affected by infertile male age. However in the control group, conventional semen parameters in addition to DNA fragmentation and chromosomal abnormalities did not show a statistically change with age.

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