Ultrastructural nuclear defects and increased chromosome aneuploidies in spermatozoa with elongated heads

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BACKGROUND: Cellular and molecular mechanisms leading to elongated sperm heads are not known. We have analysed the nuclear status of spermatozoa with elongated heads. METHODS: Fourteen men with at least 30% of spermatozoa with an elongated nucleus were studied and compared with five fertile men as controls. Sperm morphology was analysed by a quantitative ultrastructural analysis. Sperm chromosomal content was assessed by three-colour fluorescence in-situ hybridization (chromosomes X, Y, 18). Y chromosome microdeletion and karyotype were analysed. RESULTS: Elongated sperm head rates of the patients were 46.9% (30–75 versus 0–2% in the control group) by light microscopy and 34.4% by electron microscopy. In all patients, the chromatin was poorly condensed in elongated sperm heads (50% of elongated nuclei). No anomalies of sperm biochemical markers were found. All the men showed normal karyotype (46,XY) and absence of Y chromosome microdeletion. Aneuploidy rates of gonosomes and chromosome 18 were significantly increased in patients (1.64- and 3.6-fold, \( P < 0.006 \) and 0.026, respectively).

CONCLUSIONS: This study demonstrates that impaired chromatin compaction and slightly increased chromosome aneuploidies are found in spermatozoa with an elongated head, suggesting possible mechanisms such as meiotic non-disjunctions or spermiogenesis anomalies.

Key words: chromosome abnormalities/electron microscopy/elongated sperm head/fluorescence in-situ hybridization/male infertility

Introduction

The normal shape of the head of the human spermatozoon is oval, with a length of 5–6 µm and width of 2.5–3.5 µm. Long-headed sperm can have a 1-fold increased overall head length, whereas the width is slightly reduced (Dadoune et al., 1980). Consequently, the acrosome covers only 30% of the sperm head (Dadoune et al., 1980). The increased sperm head length results from an abnormally elongated nucleus that also presents particular membranous layers between the outer and inner leaves of the nuclear envelope (Rouy and Sentein, 1977). These sperm nuclear anomalies are associated with anomalies of the neck region and the persistence of a cytoplasmic droplet (Rousso et al., 2002). Most frequently, in routine analysis, spermatozoa with an elongated nucleus are recorded considering the head shape characteristics, such as tapered head (Auger et al., 1990; Urry et al., 1990) and pyriform or pear-shaped head (Kubo-Irie et al., 2005; Rousso et al., 2002), without reference to the increase in nuclear length. This type of sperm morphological anomaly never affects the whole sperm population and is present in variable proportions in the semen of the same subject (Jagoe et al., 1986). Thus, elongated-head sperm cells are present in human semen at a mean of 5.9% in fertile men (Schwartz et al., 1984) and at a mean of 22% in subfertile men (Rousso et al., 2002). A low fertilization rate has been found in men with severely elongated sperm heads (Osawa et al., 1999). The incidence of spermatozoa with an elongated head is also increased in infertile men with varicocele (Portuondo et al., 1983; Naftulin et al., 1991), with urogenital infections (Menkveld and Kruger, 1998) and in the case of workers in a reinforced plastic production (Jelnes, 1988).

The mechanisms that lead to the aberrant nuclear elongation have not been elucidated. Although an increased size of the nucleus as a whole, in addition to the increased length, has been described (Katz et al., 1986), this type of teratospermia has not been investigated at the level of the sperm chromosomal content. Other types of morphologically abnormal human spermatozoa have been found to present chromosomal...
anomalies. For example, the macronuclear spermatozoa are affected by a high level of hyperploidy (Yurov et al., 1996; Vicari et al., 2003) and particularly tetraploidy (Benzacken et al., 2001; Devillard et al., 2002; Lewis-Jones et al., 2003; Mateu et al., 2006). Whether anomalies of the chromosomal numerical content or any other molecular mechanism, such as alteration of the chromatin compaction, are responsible for the increased nuclear volume of elongated spermatozoa is questioned. We report the ultrastructural and cytogenetic studies of a series of 14 patients with semen containing spermatozoa with elongated heads.

Materials and methods

Patients and controls

The patients were referred to the Laboratory of Andrology because of a history of infertility. They were retained on the criterion of at least 30% of elongated sperm heads in two successive routine analyses. Five fertile men were included as controls. History of genitourinary infections or previous testicular injury, family infertility and personal habit (occupational hazards, drug, tobacco and alcohol intake) were recorded. They had undergone neither medical nor surgical treatment during the 3 months prior to semen examination. They had normal secondary sex characteristics with no gynecomastia. Scrotal examination was performed to confirm that testes were bilaterally descended, of normal size and of soft consistency. The patients were informed of the exact nature and the goal of the investigations performed and provided written informed consent according to French law.

Semen parameters

Semen samples were collected by masturbation into a sterile plastic cup after at least 2–7 days of ejaculatory abstinence (4 days on average). Only completely collected semen samples were included. Semen samples were allowed to liquefy for 30 min up to 1 h and were then evaluated under light microscopy, according to World Health Organization (WHO) guidelines (WHO, 1999). The recorded variables taken into consideration were volume of the ejaculate (ml), sperm concentration (×10^6 ml^-1), total sperm count (×10^9), percentage of progressive sperm cells (a + b), sperm viability (%) and percentage of morphologically normal spermatozoa. Sperm heads were considered as elongated when the head showed an increase of at least 20% of their length, including pear-shaped spermatozoa. Tapered spermatozoa with a reduced width and a normal length of at least 20% of their length, including pear-shaped spermatozoa. Sperm viability was assessed by eosin–negrosin staining. Bacterial cultures and a leukocyte count were carried out on semen samples (Leucoscreen™, Fertipro, Beerem, Belgium).

Biochemical markers were assessed on seminal plasma obtained by centrifugation at 600 g for 20 min, and deproteinization was achieved on a centrifugal filter device Ultrafree-0.5 (Millipore Corp, Bedford, MA, USA). Markers of epididymis function were carnitine (nM) and α-1-4 glucosidase (mU). Markers of the prostate secretions were citric acid (µM), prostatic acid phosphatase (U) and zinc (µM). Marker of seminal vesicles was fructose (µM).

Electron microscopy analysis

The samples were centrifuged at 350 g for 10 min and the supernatant was discarded. The sperm pellet was fixed in a solution of 2.5% glutaraldehyde (Agar Scientific Ltd, Cambridge, UK) and 0.1 mol ml^-1 sodium cacodylate/HCl/phosphate buffer pH 7.2–7.4. After centrifugation, the sample was suspended in 0.2 M sodium cacodylate buffer. Secondary fixation was performed using 1% osmium tetroxide (Agar Scientific), after which spermatozoa were dehydrated in graded alcohol and embedded in Epon resin (Polysciences Inc., Warrington, PA, USA). Semi-thin sections were stained with toluidine blue-Azur II and examined on a Zeiss Axioscope photomicroscope (Carl Zeiss S.A.S., Oberkochen, Germany). Ultra-thin sections (90 nm) were cut with a Reichert OmU2 ultra-microtome (Reichert-Jung AG, Wien, Austria) using a diamond knife, mounted on nickel grids, stained with uranyl acetate and lead citrate, and examined using a JEOL JEM 100CX II electron microscope (Jeol Ltd, Tokyo, Japan) operated at 80 kV. Photographs were taken on Kodak electron microscopy film. Negatives were scanned using an Epson Perfection 3200 photo flat bed scanner with a transparency attachment (Seiko Epson Corporation, Nagano, Japan). Digitized images were processed using Adobe Photoshop software programme (Adobe System Inc., San Jose, CA, USA). For each patient, the quantitative analysis in electron microscopy was performed on 25–30 longitudinal sections of the sperm head (including the basal plate) and on at least 50 transverse sections of the principal piece of the flagellum. The rates of chromatin condensation anomalies have been estimated, first considering the total population of sperm heads of each patient and, second, considering only the elongated sperm heads of each patient. The rate of chromatin condensation anomalies was compared with the mean value (under 22%) in normal sperm (Escalier 1983a, 1990).

Genetic analyses

The constitutional blood karyotype of the patients was performed on cultured lymphocytes, according to standard techniques. Microdeletion analysis of the Y chromosome used a sequence tagged sites (STS)-PCR technique. Nineteen STS corresponding to the three distinct AZF loci were selected. The STS tested were sY84, sY95, sY124, sY143, sY130, sY134, sY136, sY152, sY240, sY232, sY249, sY156, sY204, sY208, sY254, sY269, sY158 and sY160.

Fluorescence in-situ hybridization

A three-colour fluorescence in-situ hybridization (FISH) analysis was performed, using probes specific for chromosomes 18, X and Y. Semen from fertile patients was used as controls. After liquefaction at room temperature for 60 min, 1 ml of neat semen was diluted in 1 ml of Ferticult™ (Fertipro). After centrifugation at 500 g, the pellets were fixed on slides with freshly prepared Carnoy’s fixative solution (methanol–acetic acid, 3:1). Sperm nuclei were decongested using NaOH solution (1 mol L^-1) for 2 min at room temperature. Slides were then dehydrated through an ethanol series (70, 90, and 100%) and air-dried. Five microlitres of DNA probe mixture were deposited on the sperm nuclei preparation, covered with a coverslip and sealed with rubber cement. We used the commercial kit Aneuvision™ (Vysis, Downers Grove, IL, USA) with DNA probes specific for chromosome 18 (CEP 18 spectrumAqua™ 18p11.1–q11.1), for X chromosome (CEP X SpectrumGreen™ Xp11.1–q11.1) and for Y chromosome (CEP Y SpectrumOrange™ Yp11.1–q11.1). Denaturation was performed at 73 °C for 3 min. Hybridization was performed by overnight incubation in a dark, moist chamber. After hybridization, the coverslips were removed and slides were washed for 2 min in 0.4 × saline sodium citrate (SSC) 0.3% Nonidet P40 (NP40) solution at 73 °C, followed by 30 s wash in 2.0 × SSC 0.1% NP40 solution at room temperature. Slides were then counterstained with 4',6-diamidino-2-phenylindol dihydrochloride (DAPI; Sigma-Aldrich, Saint Quentin Fallavier, France) and mounted in antifading solution (Vectashield, Vector Laboratories, Burlingame, CA). Fluorescent signals were evaluated in 1000 spermatozoa for each sample and for each probe. FISH slides were screened...
using an X-100 objective on a Zeiss epifluorescent microscope equipped with aqua, fluorescein isothiocyanate and rhodamine single band pass filters. Only individual and well-delineated sperm nuclei were scored. We used the Martin and Rademaker scoring criteria (Martin and Rademaker, 1995). A spermatozoon was scored as disomic if it showed two hybridization signals of the same colour, size and intensity. Two spots separated by less than the diameter of one hybridization domain were scored as a single signal. The absence of hybridization signal for a single chromosome was scored as nullisomy for this chromosome only if the other probed chromosome gave a signal. No FISH signals in a spermatozoon head showing DAPI stain were considered a case of no hybridization. Sperm aneuploidy rates were determined by the sum of nullisomy and disomy rates.

The Student’s t-test, the Mann–Whitney nonparametric test and the Pearson correlation coefficient were used for statistical analyses. Differences were considered to be significant when P-value was <0.05.

Results

Clinical characteristics of the patients

Among the 14 patients, 8 patients consulted for primary infertility (patients 1, 3, 5, 7, 8, 10, 12 and 13), and 5 patients for secondary infertility (patients 2, 4, 6, 9 and 14) or tertiary infertility (patient 11). Patient 10 had diabetes and asthma. Cryptorchidism of the right testis (patient 12), varicocele (patients 9 and 12) and inguinal hernia (patient 14) were treated previously by surgery. Testes of patient 6 were injured when he was a teenager. Eight patients (patients 2, 3, 4, 5, 7, 8, 9 and 13) were smokers (>20 cigarettes per day), patient 14 was a frequent bicycle user and patient 8 presented with an occupational hazard (solvent exposure). No patient presented with heat exposure, history of genitourinary infections and neither drug nor alcohol intake. Patients 7 and 14 were from consanguineous parents.

Semen parameters

Semen parameters for each patient and control are detailed in Table I. Semen parameters of the patients group when compared with the control group were as follows (mean ± SD): total sperm count (103.3 ± 106.3 versus 419.4 ± 472.0, respectively), percentage of progressive sperm cells (21.3 ± 10.8 versus 21.0 ± 15.5, respectively), sperm viability (65.8% ± 19 versus 67.6% ± 22.8, respectively) and percentage of normal spermatozoa (4.8% ± 4.7 versus 33.2% ± 5.4, respectively). The patients presented a ‘polymorphic teratozoospermia’, where a majority of spermatozoa displayed more than one type of abnormality. Elongated sperm head rates were ranged between 30 and 75% (mean, 46.9%) in the patient group, and between 0 and 2% in the control group.

Semen biochemical markers in the patient group were found within normal range as follows (mean ± SD): creatinine (855.2 ± 555.6 mM), α-1-4 glucosidase (75.5 ± 47.7 mU), citric acid (84.8 ± 52.6 μM), prostatic acid phosphatase (104.8 ± 74.4 U), zinc (5.6 ± 3.9 μM), and fructose (62.7 ± 30.6 μM). The semen’s bacteriological cultures were negative for all patients.

Ultrastructural analysis of the sperm head characteristics of the patients

Depending on the patients, and when compared with normally elongated sperm heads (Figure 1A), 15–56% of the spermatozoa (patient 12 and 2, respectively) showed an increased nuclear length (Table II) (Figure 1B–H). Sperm heads with an increase of their length and width (Figure 1B) or both their length and thickness (Figure 1C) were considered as ‘large elongated’ sperm heads. These latter spermatozoa represented 6–31% of the whole sperm population, depending on the patients.

The nuclear content was either normally condensed (Figure 1A) or insufficiently condensed, with a granular aspect (Figure 1B and C). Considering the entire population of spermatozoa of each patient, a poorly condensed chromatin reached 32–60% (i.e. greater than the mean value for normal sperm of 22%) of the sperm heads in 8 out of the 14 patients (mean 44%) (Table II). These percentages were increased when compared with normal sperm (mean, 22%) (see Materials and methods). An insufficient chromatin condensation could be found in nuclei with a normal length (Figure 1D), but was more frequent in elongated sperm heads (Table II). Indeed, all the studied patients presented an incomplete chromatin condensation in the elongated sperm heads and at a mean of 50% (range 27–71%) (Table II). Nuclear lacunae devoid of chromatin were present in the nuclei with a normally condensed chromatin (Figure 1A) and those with granular chromatin (Figure 1B). In elongated sperm heads, the posterior region of the nucleus showed a characteristic membranous proliferation of the nuclear envelope (Figure 1B, C and E), often more developed on one nuclear side (Figure 1B).

Binucleated sperm cells were present in 11 out of the 14 patients at various percentages (range 5–27%), depending on the patients (Table II) and most of them appeared immature and in a large cytoplasm. Chromatin of these double nuclei was frequently poorly condensed (Figure 1E). Biflagellated spermatozoa were less frequent (not shown).

At the flagellum level, the patients presented with a mitochondrial sheath misassembly (all patients, range 43–89%), an absence of axonemal doublets (11 patients, range 33–58%) and various anomalies of the disposition of the ribs or of the longitudinal columns of the fibrous sheath (12 patients, range 23–77%) (data not shown).

Genetic and FISH analyses

All the men showed a normal karyotype (46,XY) and an absence of Y chromosome microdeletion. Sperm FISH results, including disomy and nullisomy rates, are indicated in Table I. The aneuploidy rates for chromosome 18 varied from 0 to 1.6% in the patient group (mean, 0.93%) and from 0.1 to 0.5% in the control group (mean, 0.26%). The aneuploidy rates for gonosomes varied from 0.6 to 3.4% in the patient group (mean, 1.78%) and from 1.1 to 1.3% in the control group (mean, 1.08%). Aneuploidy rates of gonosomes and chromosome 18 were significantly higher in patients with elongated sperm heads when compared with the values of control sperms (1.64- and 3.6-fold increase, P = 0.006 and P = 0.026, respectively). Mean diploidy rates were 0.26% in the patient group and 0.20% in the control group and not significantly different between the two groups.

Spearman analyses between gonosomes or chromosome 18 aneuploidy rates, and sperm parameters (total sperm count,
Table I. Sperm parameters, as assessed by light microscopy, and fluorescence in-situ hybridization results in patients with elongated sperm heads and in control men

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<th>Elongated heads (%)</th>
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</table>
This hidden nuclear posterior region does not allow the estimation of the real extension of the nucleus of spermatozoa, as observed by light microscopy. Conversely, electron microscopy did not allow the estimation of the whole nuclear shape since the ultra-thin sections allow the observation of only 2D of the sperm nuclei (i.e. the length and either the width or the thickness).

Most frequently, in routine analysis by using light microscopy, spermatozoa with an elongated nucleus are recorded considering the head shape characteristics, such as tapered head (Auger et al., 1990; Urry et al., 1990) and pyriform or pear-shaped head (Rousso et al., 2002; Kubo-Irie et al., 2005), but without reference to any increased nuclear length. Only two publications studied elongated-head spermatozoa by using electron microscopy (Rouy and Sentein, 1977; Dadoune et al., 1980). Using scanning electron microscopy, Dadoune et al. (1980) differentiated thin and elongated sperm head (long-headed sperm). This study showed that the acrosome accounted for 30% of the elongated sperm head, the post-acrosomal cap, the posterior nuclear space, for 9 and 6%, respectively, and the posterior part of the head covered with a voluminous cytoplasmic sheath for 56%. Rouy and Sentein (1977) performed a study by transmission electron microscopy of elongated spermatozoa both in semen and in a testicular biopsy. They found particular membranous layers between the outer and inner leaves of the nuclear envelope in the posterior nuclear region that formed during the spermiogenesis. All these morphological anomalies were present in the elongated spermatozoa of our study. Also a coiled flagellum is frequently present in spermatozoa with an elongated sperm head that could be explained by anomalies of the localization or size of the basal plate as a result of the membranous proliferation. It is worth noting that the percentage of progressive spermatozoa is low in all the studied patients. The associated anomalies of the sperm head and flagellum should contribute to the motility defects. A previous study showed that morphologically normal spermatozoa swam faster and straighter and exhibited higher beat frequencies than did morphologically abnormal sperm and that the differences were most pronounced for spermatozoa with elongated or pyriform tapering heads (Katz et al., 1982). Importantly, the chromatin was poorly compacted in a high percentage of elongated sperm heads, suggesting that the last step of chromatin maturity is impaired. Immaturity of the chromatin in elongated sperm heads has been already described by using aniline blue staining (Auger et al., 1990). Also, we observed no anomalies in semen zinc or fructose levels, which are considered by several authors (Kvist et al., 1987; Bjorndahl and Kvist, 1990) as a possible mechanism involved in chromatin condensation. The rate of elongated sperm heads with anomalies of chromatin condensation is close to that of macronuclear spermatozoa (Escalier, 1983b).

In our study, aneuploidy rates, assessed by FISH, were statistically higher in patients with elongated sperm heads than in controls. Percentages of aneuploid sperm are higher in cases of impaired sperm parameters: oligozoospermia (Gianaroli et al., 2005; Nagvenkar et al., 2005; Pang et al., 2005; Rives, 2005), asthenospermia (Bernardini et al., 2005; Templado et al., 2005).
and teratospermia (Machev et al., 2005; Templado et al., 2005), including structural flagellar anomalies (Carrell et al., 2004; Rives et al., 2005). Only two types of spermatozoa with anomalies of the nucleus have been investigated by FISH. In the case of round headed spermatozoa (also called globozoospermia), anomalies of the chromosome content have been found at various degrees, depending on the patients (Carrell et al., 1999, 2001; Martin et al., 2003; Morel et al., 2004; Ditzel et al., 2005). The macronuclear spermatozoa have been found to present with a high level of hyperploidy (Yurov et al., 1996; Vicari et al., 2003), particularly tetraploidy (Benzacken et al., 2001; Devillard et al., 2002; Lewis-Jones et al., 2003; Mateu et al., 2006). A report on FISH analysis of men with either large or elongated sperm nuclei (only a published abstract available) indicated a hyperploidy reaching 23.4% (Fedorova, 2003). This high level of hyperploidy suggests that the patients analysed shared a particular sperm phenotype such as elongated sperm head originating from germ cells with impaired meiotic divisions (i.e. macrocephalic). In our study, the increased rates of aneuploidy were in the same range as those found in men with either oligospermia, or teratospermia, or asthenospermia (Gianaroli et al., 2001, 2003; Martin et al., 2001; Devillard et al., 2003). Spermatids were found wrongly positioned to Sertoli cells and Sertoli–germ cell junction complexes structurally abnormal (Cameron et al., 1980). Besides, it is interesting to note that elongated sperm heads have been found associated to an abnormally narrow shaped spermatid microtubular manchette (Rouy and Sentein, 1977), as also previously observed in the azh mutant mouse model (Cole et al., 2006). This suggests that the manchette could exert an excessive pressure on the nucleus before its condensation and could explain why elongated sperm heads frequently present an elongated and tapered nucleus.

Interestingly, rat testes with an experimental varicocele present with a diminution of the expression of Fas (but not of FasL) (Celik-Ozenci et al., 2006) and of Notch-1 and -2 (Sahin et al., 2005). Fas has been found in cytoplasmic extrusions of elongated spermatids and it was suggested that Fas might be necessary for normal spermigenesis, especially for normal sperm head morphology (Celik-Ozenci et al., 2006). Notch signalling is known to control cell fate through local cell interactions. Notch-1 is expressed in elongated spermatids and may be related to their maturation (Sahin et al., 2005). Moreover, men with varicocele have been found to have an ectopic nuclear localization of the C3 subunit of the 20S

Table II. Ultrastructural anomalies of sperm nuclei in patients with elongated sperm heads (%)

<table>
<thead>
<tr>
<th>Patients</th>
<th>Nuclear shapes</th>
<th>Nuclear number anomaly</th>
<th>Chromatin insufficient condensation</th>
<th>Nuclear envelope</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Elongated (total) Elongated Elongated and large Short or irregular</td>
<td>Binucleated Total</td>
<td>Elongated nuclei</td>
<td>Membrane proliferation</td>
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<tr>
<td>1</td>
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<td>48</td>
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<tr>
<td>14</td>
<td>23</td>
<td>17</td>
<td>6</td>
<td>64</td>
</tr>
</tbody>
</table>

*Chromatin condensation was estimated considering all the sperm heads.

*bChromatin condensation was estimated considering elongated nuclei only.

In varicocele patients, the percentage of ‘tapered’ sperm heads is variably estimated to reach 20 (Portuondo et al., 1983) or 36% (Naftulin et al., 1991), and these patients present an increase in sperm numerical chromosome anomalies (Baccetti et al., 2006). In our study, patients 9 and 12 had a previously cured varicocele. Their sperm numerations were normal and both showed a rate of aneuploidy in the mean range of the studied patients (Table I).

The mechanisms leading to an excessive elongation of the sperm nucleus are poorly understood. Only testes from cases with varicocele and elongated sperm heads have been investigated. The scrotal temperature was found to be higher in infertile men with a varicocele (Ali et al., 1990; Naughton et al., 2001), and Sertoli cells were found to be injured (Terquem and Dadoune, 1981). Spermatics were found wrongly positioned to Sertoli cells and Sertoli–germ cell junction complexes structurally abnormal (Cameron et al., 1980). Besides, it is interesting to note that elongated sperm heads have been found associated to an abnormally narrow shaped spermatid microtubular manchette (Rouy and Sentein, 1977), as also previously observed in the azh mutant mouse model (Cole et al., 1988). This suggests that the manchette could exert an excessive pressure on the nucleus before its condensation and could explain why elongated sperm heads frequently present an elongated and tapered nucleus.
proteasome in their spermatozoa (Ziemba et al., 2002). These recent data open the door towards interesting investigations in order to define which nuclear molecular factor(s) could be involved in the abnormal sperm nuclear elongation and meiotic division errors.

In conclusion, our study demonstrates that the chromatin compaction is altered in spermatozoa with an elongated head and that the numerical chromosome content is slightly, but significantly, disturbed. These data should lead further studies to identify the mechanisms behind the meiotic errors and chromatin compaction impairment in patients with elongated sperm heads.

References


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