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Original Article

Differences in caspase-8 and -9 activity and sperm motility in infertile males of Li nationality in China

Xiaobin Wei[1,2], Qixing Li[2], Zhouxin Han[3], Danqin Lin[3], Ping Yu[1]

[1]Department of Immunology, College of Basic Medical Sciences, Central South University, 88 Xiangya Road, Changsha 410008, China; [2]Clinical Laboratory, Affiliated Haikou Hospital of Xiangya Medical College in Central South University, 43 People's Blvd, HaiKou 570208, China; [3]The Second people's Hospital of Hainan Province, 24 Aimin Road, Wuzhishan 572200, China

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Abstract: This study's objectives are to assess the efficacy of detecting apoptotic caspase-3, -8, and -9 in human sperm and plasma using enzyme-linked immunosorbent assays (ELISA), and to compare these levels between fertile and infertile patient groups of Li nationality in China. This study offers a non-invasive, alternative strategy to analyzing sperm parameters in infertile males. Fifty-six infertile males were investigated; asthenospermia (n = 19), oligoasthenoteratozoospermia (n = 20), azoospermia (n = 17) compared with 20 healthy fertile controls. They were subjected to semen analysis by computer-assisted sperm assay (CASA). We found that caspase-3, -8, -9 existed in all specimens in both sperms and plasma. The level of caspase-3 and caspase-8 in plasma were both significantly higher than in sperm. Levels of caspase-8 and caspase-9 in sperm and plasma were significantly negatively correlated with sperm concentration, motility and A % (motility grade A). The level of caspase-8 in plasma was significantly negatively correlated with sperm concentration. However, only in healthy fertile controls sperm concentration was significantly negatively correlated with caspase-9 in sperm. Compared with the healthy fertile controls, only the OAT group exhibited significantly increased level of caspase-8 in sperm ($P < 0.05$). It is concluded that caspase-8 and caspase-9 in sperm and plasma are correlated with sperm motility, and can reflect the quality of sperm in vitro.

Keywords: Sperm, apoptosis, caspase, Li nationality

Introduction

Infertility is caused by various reasons, and its pathogenesis is also complicated. Caspase is a hot topic in studies on infertility, and its relationship with etiology and pathogenesis of infertility has been proved by several researches [12, 13]. Caspase, DNA and mitochondrial membrane are regarded as routine indexes of apoptosis. And etiology of male infertility seems to be closely associated with density, motility and morphology of sperm [13]. Caspases play a central role in the regulation of apoptosis in the human seminiferous epithelium. They are expressed as inactive proenzymes and participate in a cascade triggered in response to pro-apoptotic signals. To date, 14 caspases have been implicated in the human apoptotic pathway cascade. Among receptor-mediated programmed cell death (PCD), caspase-3 is considered to be a major executioner protease,caspase-8 plays the most important role in transduction of death signals and Activated Caspase-9 cleaves downstream caspases such as Caspase-3, -6 and -7 initiating the caspase cascade [18]. So, we focus on caspase-3, -8 and -9.

As a boundary island between subtropical and tropical zones, Hainan province, China, has a unique climate and unique geographical conditions. The Li people are a minority, with characteristic genetic background. The Li people are centralized within Hainan province and are spread throughout the province, but mainly live in the central and southern regions, with an estimated population of 1.2 million [27]. Traditional Li villages are based on clanship and kinship relationships that embody the region's cultural style and have developed over many years. For a long time, infertility has been the main disease that has troubled the Li people. About 15% of the married couples of child-bearing age in this population are infertile, among

Table 1. CASA results ($\bar{x}$ ± s)

Group	*n*	Density (10^6/mL)	Motility (%)	A %
Healthy fertile controls	20	106.71 ± 52.80	63.18 ± 7.56	37.05 ± 8.38
Infertility group I	19	82.54 ± 42.21	42.53 ± 11.05[a]	12.41 ± 6.91[a]
Infertility group II	20	9.21 ± 5.48[a]	51.08 ± 19.86[a]	2.68 ± 5.05[a]
Infertility group III	17	0[a]	0[a]	0[a]

[a]Significant difference compared with the controls.

which 20%~30% of the cases may be associated with immunologic factors [4]. However, the scientific basis for the infertility etiology of the Li people has not been elucidated.

Nowadays, besides physical examination and conventional semen examination, testicular biopsy is usually applied to evaluate production and development of sperm in infertility diagnosis. This study aims to explore a non-invasive, safe, economical and reliable method for infertility diagnosis. We compared levels of caspase-3, -8, -9 in plasma and in sperm between healthy males and infertile males of Li nationality, and analyzed the relationship between sperm motility, density and the level of caspase-3, -8, -9 to explore pathogenesis of oligoasthenozoospermia.

Materials and methods

A total of 56 infertile males of Li nationality (aged 25-40 years old) were recruited prospectively from Andrology Department of The Second people's Hospital of Hainan Province after the Hospital Ethics Committee approval. They were divided according to their semen analysis into asthenospermia (n = 19), oligoasthenoteratozoospermia (n = 20), azoospermia (n = 17) compared with 20 healthy fertile controls of Li nationality (aged 28-37 years old), fathering children, of matched age. Exclusion criteria were varicocele, leukocytospermia, diabetes, collagen diseases, liver disorder, tumours, low semen volume, sperm agglutination and cases under anti-arthritic or hemolytic drugs [23]. They were subjected to full history, physical examination and genital examination. All cases participated in this study voluntarily. More than one semen samples were collected by masturbation after sexual abstinence for 3-5 days to verify the diagnosis and were examined according to World Health Organization guidelines (WHO, 2010) by computer-assisted sperm assay (CASA) method. This tablet generates data that can be analyzed by a microcomputer programme to calculate sperm concentration, percentage of motile sperm, average curvilinear velocity and linear velocity. Objective classification of motility: grade A linear velocity > 22, grade B < 22 and curvilinear velocity > 5 μm s^{-1}, grade C curvilinear velocity < 5 μm s^{-1} and grade D immotile spermatozoa. The healthy donor specimens had the following criteria: sperm concentration > 15 million/ml, > 32% progressively motile (WHO a + b) and > 4% with a normal morphological shape (WHO, 2010).

Caspase-3, -8, -9 were quantitatively determined by ELISA (agents were provided by R&D System Co.). Specimens, standards, HRP-labeled antibodies were added successively into micropores which were pre-coated with antibody captured by caspase-3, -8, -9. These micropores were incubated and washed completely. Substrate TMB was used for developing. TMB became blue under catalysis of peroxidase, and then finally turned out to be yellow under the activity of acid. The intensity of the color is measured at 450 nm using a spectrophotometer. In order to measure the concentration of caspase-3, -8, -9 in the sample, this caspase-3, -8, -9 ELISA Kit includes a set of calibration standards. The calibration standards are assayed at the same time as the samples and allow the operator to produce a standard curve of Optical Density versus caspase-3, -8, -9 concentration. The concentration of caspase-3, -8, -9 in the samples is then determined by comparing the O.D. of the samples to the standard curve. All were processed strictly according to instruction.

Statistical analysis

The data were computerised and checked for consistency. Analysis of results was performed using SPSS, version 16.1 (SPSS Inc., Chicago, IL, USA). Data were represented as mean ± SD. The enzyme level among groups were compared by DUNNET-*t* test; the relationship between enzyme level and routine sperm parameters was analyzed by Pearson linear correlation analysis; the relationship enzyme level

Table 2. Caspase-3,-8,-9 levels ($\bar{x}$ ±s, pmol/L)

Group	*n*	caspase-3		caspase-8		caspase-9	
		In plasma	In sperm	In plasma	In sperm	In plasma	In sperm
Healthy fertile controls	20	7.55 ± 7.78	6.32 ± 3.76	14.99 ± 12.93	9.85 ± 5.57	7.01 ± 6.10	5.76 ± 2.33
Infertility group I	19	8.77 ± 9.13	5.19 ± 4.91	16.88 ± 12.13	10.70 ± 2.24[a]	9.28 ± 9.07	7.13 ± 7.25
Infertility group II	20	8.59 ± 8.71	7.52 ± 8.80	16.54 ± 7.17[a]	16.04 ± 10.63[a]	8.12 ± 6.19	9.22 ± 5.28
Infertility group III	17	11.44 ± 11.50	6.34 ± 2.76	21.48 ± 11.80	15.71 ± 6.46	8.28 ± 3.38	10.65 ± 7.34

[a]Significant difference compared with the controls.

Table 3. Correlation between Caspase and sperm parameters

Sperm parameters	Plasma			Sperm		
	caspase-3	caspase-8	caspase-9	caspase-3	caspase-8	caspase-9
Density	NC	*r* = -0.288 *P* = 0.012	NC	NC	*r* = -0.324 *P* = 0.007 *r* = -0.632 *P* = 0.000	*r* = -0.241 *P* = 0.046 *r* = -0.557 *P* = 0.000
Motility (%)	NC	NC	NC	NC	*r* = -0.343 *P* = 0.004	*r* = -0.283 *P* = 0.018
A%	NC	*r* = -0.326 *P* = 0.004	*r* = -0.241 *P* = 0.036	NC	*r* = -0.351 *P* = 0.003 *r* = -0.673 *P* = 0.000	*r* = -0.316 *P* = 0.008 *r* = -0.490 *P* = 0.000

NC: no correlation.

and kinetic parameters was analyzed by Spearman's rho. Statistical significance was set at $P < 0.05$.

Results

CASA results

All sperm parameters were in standard range of WHO (WHO, 2010). Mean (motility grade A) A % was lower than 15% in infertility group I (oligo-asthenoteratozoospermia group), which was significantly different from that of healthy fertile controls ($P < 0.001$); Mean A % was also lower than 15% in infertility group II (asthenospermia group), and the mean density was lower than 15×10^6 /mL, both of which were significantly different from those of healthy fertile controls (both $P < 0.001$); the density, motility and A % were 0 in infertility group III (azoospermia group), which were all significantly different from those of healthy fertile controls (all $P < 0.001$) (**Table 1**).

Caspase-3, -8, -9 levels

The results revealed caspase-3, -8, -9 in both sperm and plasma of all groups. In infertility group I, the mean level of caspase-3 and caspase-8 [(16.88 ± 12.13) pmol/L *vs.* (10.70 ± 2.24) pmol/L, $P < 0.05$] in plasma were both higher than these in sperm; while in infertility group II and III, the mean level of caspase-3, -8 and -9 in plasma were all higher than these in sperm. Compared with healthy fertile controls, the mean level of caspase-8 and -9 in sperm and plasma of infertility group I, and caspase-3, -8 and -9 in sperm and plasma of infertility group II and III were all higher. Caspase-8 in infertility group II was significantly higher than that of healthy fertile controls (16.04 ± 10.63) pmol/L *vs.* (9.85 ± 5.57) pmol/L, $P < 0.05$] (**Table 2**).

Correlation between caspase and sperm parameters

The analysis showed that mean levels of caspase-8 and-9 in sperm were negatively correlated with sperm density (caspase-3: $r = -0.324$, $P < 0.05$; caspase-8: $r = -0.241$, $P < 0.05$), and were negatively correlated with sperm motility (caspase-3: $r = -0.343$, $P < 0.05$; caspase-8: $r = -0.283$, $P < 0.05$). caspase-8 and-9 in plasma and sperm were both negatively correlated with A % (In plasma caspase-3: $r = -0.326$, $P < 0.05$; caspase-8: $r = -0.241$, $P < 0.05$. In sperm caspase-3: $r = -0.673$, $P < 0.05$; caspase-8: $r = -0.490$, $P < 0.05$). Other parameters had no correlation with Caspase in sperm/plasma (**Table 3**).

Discussion

This study showed caspase-3, -8, -9 exist in plasma, caspase-8 and caspase-9 in sperm

and plasma in infertile males of Li nationality are correlated with sperm motility, and can reflect the quality of sperm in vitro. Apoptosis refers to programmed cell death. It is necessary for maintaining normal development of tissues and organs, and cleaning abnormal cells. Besides, it plays an important role in sperm differentiation and testicular maturity in mammalian testicular tissues. The full name of caspase is aspartic acid specific cysteine proteinases and it is a principal member in apoptosis mechanism.

At present, there are 14 kinds of known human caspase. They cause cascade reaction to mediate apoptosis via two pathways: membrane death receptor pathway and mitochondrial pathway. During these pathways caspase-8 [12] and caspase-9 are the most vital promoters, while caspase-3 is the execution factor during common pathway [6]. This study detected these three caspases to explore apoptosis mechanism, which is associated with casepase, sperm motility and sperm density.

In regards to male reproduction, several studies on caspase have been reported. At firstly they focused on existence of apoptosis markers of somatic cells in testis and epididymis, then they put emphasis on relationship between testis and caspase under different pathological conditions [8, 10, 19, 22], followed by existence of apoptosis markers in sperm *in vitro* [3, 16]. At present some researches pay attention to correlation between caspase and sperm under specific pathological and physiological conditions [17, 20], and some focus on the effect of various factors on sperm apoptosis markers *in vitro* [7, 25]. These studies are helpful in exploring etiology and pathogenesis of infertility, and in application of in-vitro fertilization (IVF)/intracytoplasmic sperm injection (ICSI). They also provide scientific evidence for treatment of male infertility.

Many semen processing methods *in vitro* have certain influence on sperm or reproductive cells, such as centrifugal force during mechanical processing [2], magnetic field force during MACS separation [2], refrigeration under deep hypothermia [25], density gradient centrifugation, swim-up method *etc* [1, 15]. So it is difficult to determine if caspase is activated before or after ejaculation or at both time [11, 28]. On the condition that caspase exists in plasma, the influence due to processing *in vitro* can be ignored, and apoptosis status of sperm before ejaculation can be reflected directly. Caspase isolated from sperm can reflect apoptosis after ejaculation. Considering that healthy sperm cannot be in apoptosis spontaneously after ejaculation [11, 14], and they always died of necrosis not of apoptosis [11], this apoptosis may be due to hypoplasia of few sperm initiating in testis or epididymis, or due to activation of caspase during *in vitro* processing. It is reported that caspase exist in testis and epididymis [8, 10, 19, 22], so residue of apoptosis cells would exist in semen and be discharged, which provide explanation for existence of caspase in plasma. It is also reported no activated caspase can be detected in supernatant after centrifugation [3], but we have detected caspase in semen specimens of azoospermia patients.

In our study, caspase-3, -8, -9 existed in all specimens of Li nationality people, which is consistent with most reports. Some researchers have detected proenzyme of caspase-3 and -9, and lowly activated caspase-3 and -9 in patients and healthy persons by immunoblotting [21]; some found caspase-3, -8 and -9 both in proenzyme and activation form [25]; and some reported existence and activation of caspase-3, -8, -9 in sperm [7]. This study showed that compared with healthy fertile controls mean level of caspase-8 and -9 in asthenospermia group, caspase-3, -8, -9 in oligoasthenoteratozoospermia group and azoospermia group were higher.

We detected caspase-3, -8 and -9 in all specimens, which supported existence of caspase in plasma. It can also be presumed that residue apoptotic cell of testis and epididymis tissues exist in plasma *in vitro*, and these residue can reflect apoptosis status of testis and epididymis tissues to some degree. Besides, caspase in plasma can also reflect other function of caspase. It is assumed that proenzyme form of caspase may be associated with other function except apoptosis, especially during maturity and development of sperm [5, 9, 26]. In addition, the effect of caspase in plasma on alive or apoptotic sperm still need further study.

In conclusion, caspase-3, -8, -9 exist in plasma of Li nationality people, and caspase-8 & -9 can reflect sperm parameters *in vitro* such as A %. More importantly, caspase -8 & -9 can reflect cell apoptosis status in testis or epididymis.

Caspase-8 & -9 are associated with sperm parameters such as density, motility and A %, and they are favorable indicators of sperm quality.

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Disclosure of conflict of interest

None.

Address correspondence to: Ping Yu, Department of Immunology, College of Basic Medical Sciences, Central South University, 88 Xiangya Road, Changsha 410008, China. Tel: t86-731-4487301; Fax: t86-731-4487301; E-mail: yuping1953@sina.com

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