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The plasmatic oxygen radical antioxidant capacity (*ORAC*) index but not the neutrophil enzyme Lcn2 level may predict high levels of semen malondialdehyde in male idiopathic infertility

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ABSTRACT

Male infertility is a factor in 50% of cases but no cause can be diagnosed in approximately 25% of infertile males. This is termed 'idiopathic infertility' and is thought to be linked to reduced antioxidant defense. The present study aimed to find a possible relationship between reducing antioxidant capacity, malondialdehyde (MDA) formation, and the compensatory antioxidant system of the neutrophile gelatinase associated lipocaline (NGAL) level and the standard semen parameters in idiopathic infertility. Thirty five Algerian soldiers including fertile men (n=8) and infertile men (n=27) who were selected for the heat stress and extreme physical exercises at least for 2 years in desert military units. The oxidative stress (OS) balance, total antioxidant capacity (TAC) versus MDA, was evaluated by fluoremetric methods either in semen and plasma samples. The plasmatic NGAL was measured by immunofluoremetric assay. Standard evaluation of seminogram was carried according to WHO guidelines. In semen, although MDA was significantly higher (p=0.000) while TAC were significantly lower (p=0.003) in infertile men than fertile men, no correlation was found between standard sperm parameters and MDA or TAC. In spite of the significant difference of plasma TAC between the two groups (p=0.000), no difference among them for MDA was detected (p=0.411). The semen TAC was 7.96 folds higher than the plasma one and the plasma level of MDA was higher by 1.05 folds than the seminal one. No difference between patient and control plasma NGAL level was found. We noticed a strong correlation between plasma ORAC decreasing and MDA generation in semen (r=-0.753, p=0.000). Consequently, we have determined a cut-off of 1.035 m mol eq AA/l for the antioxidant defense level that predict semen MDA generation (Se=100%, Sp=94.4%; AUC=0.765, p=0.000). Data of the present study suggest that the plasmatic compensatory antioxidant enzyme NGAL is not associated with OS related infertility while the plasma ORAC may predict a high amount of semen toxic byproduct MDA. Furthermore, our data show no significant relathionship between semen parameters and the OS damage unless this latest may identify a part of the idiopathic infertility and may be included in routine chek-up.

Key words: Idiopathic infertility, total antioxidant capacity, malondialdehyde, NGAL, sperm parameters

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INTRODUCTION

Since the *Poly Matzinger* theory arguing that the immune system (IS) respond the danger signal regardless of its origin, self or not self, the antioxidant system starts to gain the lion part as an interface in the danger-IS interactions. This novel conception revealed pathophysiology basis of several inflammatory disorders that are currently

considered as related to oxidative stress (OS) like Alzheimer, diabetes, degenerative and cardiovascular diseases, cancers and infertility. Of note, male infertility is a relatively common condition affecting approximately 1 in 20 of the male population. Even though several conditions may cause infertility, such as obstructive, hormonal, and immunologic pathologies and varicocele; no identifiable causes can be found in more than 25% of infertile males, and these cases are considered as idiopathic infertility [1-2]. Interestingly, the major cause of defective sperm function is OS, which not only disrupt the integrity of sperm DNA but also limits the fertilizing potential of these cells by a direct damage to membrane's proteins and lipids. Historically, the fact that OS might be a factor in the aetiology of defective sperm function in humans was advanced independently, thirty years ago, by *Aitken and Clarkson, and Alvarez et al.* [3-4]. Needless to reminder that abundance of unsaturated fatty acids (PUFA) in sperm membrane that have a high affinity of reactive oxygen species (ROS) are determinant in maintaining the oxidant and antioxidant balance during sperm normal functions [5-7]. Nevertheless, such bio-molecules (e.g. lipids) are considered to be the most susceptible macromolecules to ROS attacks, and may initiate a dangerous cascade of chemical reactions called *lipid peroxidation* [8-14]. The major byproduct of these reactions is malondialdehyde (MDA) that cause sperm DNA damage; and in fact, is considered as the specific marker to monitor oxidative damage degree [11-12]. Furthermore, any excess of ROS could cause structural anomalies in sperm cells and subsequent reduction in sperm-oocytes conjugation capacity, and reduction in the fertilization rate [13-14]. Importantly, three different antioxidant protection systems play important and interdependent roles in reducing such ROS in males: dietary antioxidants, endogenous antioxidants, and metal-binding proteins which make this type of infertility reversible by using dietary antioxidants, usually present in the form of vitamin C, vitamin E, beta-carotenes, carotenoids, and flavonoids[17-22]. Otherwise, cumulative evidence demonstrated the existence a compensatory anti-OS responses as an ultimate mechanism of surviving against intensive ROS. Lipocalin-2, also known as Neutrophil gelatinase associated lipocaline (NGAL) is the most characterized compensatory molecule induced by OS and acts as a cytoprotective factor against H_2O_2 toxicity [23-27].Moreover, the up-regulation of Lcn-2 expression by ROS both *in vitro* and *in vivo* can exert a protective role against OS by inducing of heme oxygenase-1 (HO-1); This, indicates its potential as a surrogate measure for OS [27-29]. Generally, the major producers of ROS like chemicals that increase Lnc2 gene expression [30-37]. Finally, we should stress that semen leukocytes are associated with further OS and may impair infertility [38-44].

In the last decade, there has been a phenomenal growth in our knowledge of male reproduction, sperm function and development of diagnostic tools and treatment modalities but no study has assessed the implication of an antioxidant compensatory system in this field. Lcn2 has been proposed as a useful biomarker to assess OS both *in vitro* and *in vivo* in many conditions [27]. In this work, we sought to determine a relationship of NGAL within the total antioxidant molecules and sperm toxicity in human male idiopathic infertility.

MATERIALS AND METHODS

The institutional ethical committee clearance was obtained for the present study, on due orientation about the nature of study, a written consent was obtained from healthy individuals and infertile male subjects. It was realized in the unit of immunology of the military university hospital of Oran HMRUO, Algeria.

Subjects: This prospective study included 35 of 42 consecutive male patients attending the gynecology ward of our medical university for Assisted Reproduction Techniques. The seven excluded ones had not a minimal passage of 2 years in Sahara military units. At first clinic attendance a complete medical history and physical examination that focused on the secondary sex characteristics and congenital and/or acquired genital malformations on both husband and wife. Eight age-matched healthy males who had fathered a child in the previous year and having sperm count more than 20 million/ml with motility more than 50% in forward progression were selected from some volunteers among our laboratory personnel and considered as fertile control group according to World Health Organization (WHO) criteria [45].

Each patient's hormone profile was studied, including FSH, LH, testosterone, and prolactin. In cases where additional malformations were suspected and the physical examination was insufficient or suspicious, scrotal, transrectal, and/or abdominal ultrasonography were performed. Infertility cases related to known causes, such as varicocele, leukocytospermia, hormonal, and/or obstructive pathologies were excluded from the study. The remaining patients were regarded as idiopathically infertile. Men were excluded if they had been infertile for less than 1 year.

Semen samples: Samples were collected and analyzed as described below. Two semen samples were taken from each patient at an interval of between 1 and 3 weeks. When a 20% difference at the sperm concentration, motility, and/or morphology was found between the 2 samples, a third sample was taken. Semen samples were produced by masturbation and collected into clean glass containers after minimum of three days of abstinence. Aliquots of liquefied semen were centrifuged at 150x*g* for 20 min and their supernatant parts were stored at –80 °C to study their total antioxidant capacity (TAC) and total lipid peroxides levels (TP).

Blood samples: The participants, who had fasted for 10-12 h, were placed in a supine position and blood samples of approximately 5cm^3 were withdrawn from a cubital vein into heparinized tubes. All blood samples were taken in the morning, between 08:00 and 10:00 hours. Blood samples were immediately centrifuged at *150*x *g*, for 10 min. The plasma were separated and stored at –80 °C until analysis. TAC and TP levels were measured at the same time for all samples.

Table 1. Eligibility inclusion Criteria

	Eligibility Criteria
i.	Having worked in Sahara Military Facilities over 2 years
ii.	aged above 20 or below 45 years
iii.	no clinic varicocele
v.	no hormonal disorder
v.	no urogenital infection
vi.	no intake of multivitamin drugs in the previous 2 weeks

Seminograme: Including the volume of ejaculate (ml), sperm concentration (×10^6 /ml), forward motility (%), and morphology (% of atypical forms) after semen liquefaction were performed according to WHO guidelines [45]. Samples were stored at 37°C and analyzed within 2 hours, by the same biologist. Sperm morphology was evaluated with papanicolaou colored semear. Number and movement were evaluated using a *Lieca optic* microscope. Sperm motility was classified into 4 classes: “a” rapid progressive motility, “b” slow progressive motility, “c” non-progressive motility, and “d” no motility.

Total antioxidant capacity ORAC assay: Total antioxidant capacity levels of stored blood plasma and seminal plasma samples were measured with oxygen radical absorbance capacity (ORAC) with a 1:150 and 1:800 dilution respectively. Manual ORAC analysis was performed on a Perkin *Elmer* spectrofluorometer *Victor X* with a fluorescent filters (Ex: 485 nm; Em: 520 nm). In the final assay mixture (200 µl total volume) Fluoresceine-Na FL (12.5 nM) was used as a target of free radicals attack, with H_2O_2-Cu^{2+} (H_2O_2 0.3%; $CuSO_4$ $5H_2O$, 0.9 mM) as mainly a hydroxyl radical generator (ORAC-OH° assay). The spectrofluorometer was programmed to record the fluorescence of *FL* every 30 sec after H_2O_2-Cu^{2+} were added for as long as 35 min and the samples were thermostated. All fluorescence measurements were expressed relative to the initial reading. Final results were calculated using the differences of areas under the *FL* decay curves between the blank and a sample and expressed as millimoles of ascorbic acid equivalent per liter.

Total lipid peroxides: Malondialdehyde (MDA) levels of blood and semen plasma samples were measured by thiobarbituric acid method based on the colorimetric reaction with forming pink color product, which can be measured by spectrophotometer at a wavelength of 532 nm and reported as mmol H_2O_2/l [46]. The initial sample volume used in this assay was 150µl for both plasma and semen.

Plasmatic neutrophil gelatinase-associated lipocalin: by immuno-Fluore-chromaography tests using *Triage®Meter Platform* (*Biosite International Sarl, and Morges, Switzerland*) that specifically detect human NGAL. The assay was performed as per manufacturer’s protocol. Briefly, 250 µl of sample is applied into the device which will be inserting into the *Triage Meter Pro* and the result can be printed directly from the meter in 15 minutes and reported in ng/ml.

Statistical analysis: Statistical analysis was performed by Statistical Package for the Social Sciences (SPSS, version 20, for Windows, Chicago, SPSS, Inc). Data was reported as mean ± SD. The comparisons between two groups were tested by unpaired Student’s t-tests for independent samples because the variables were normally distributed. A 95% confidence interval was used. Correlation between two continuous outcomes was evaluated using *Spearman* correlation coefficients. *Receiving operating curve* (ROC) was used to determine the cut off values with a given sensitivity and specificity. *p* values less than 0.05 were considered as statistically significant.

RESULTS

The study included 28 patients diagnosed according to the criteria for idiopathic infertility. The mean age of the idiopathic infertile patient group and the control group was 31.03 ± 4.94 and 31.30 ± 3.95 years, respectively. The differences between the groups were not significant statistically. The semen characteristics of the idiopathic infertile patient and control groups are shown in Figure 1 and resumed in Table 2. Sperm concentration ($\times 10^6$ /ml), percentage motility (a+b), percentage of normal forms, and volume (cc) in the patient group were significantly lower than in the control group.

TAC and TP mean values of seminal and blood plasma samples are shown in Figure 2 A for the study group and the controls. In both groups, TAC was significantly higher in seminal plasma compared to blood plasma (7.96 folds) and TP levels were also higher in seminal plasma compared to blood plasma (1.05 folds). The differences between seminal plasma and blood plasma according to the means of TAC and TP values were statistically significant in both groups. Seminal levels of MDA (2.01±0.03) was 3.52 folds higher (p=0.000) in infertile men than fertile men (0.57±0.06). Seminal levels of TAC (1.48±2.03) were significantly lower (p<0.001) in infertile men than fertile men (2.66±1.02) (Table 3).

However, the means of TAC and TP values of blood plasma samples were not significantly different between the patient and control groups. Plasma NGAL level is higher in infertile men than control group but not significant statistically. Moreover, this enzyme does not correlate neither with plasmatic ORAC nor the semen one (Fig. 3A). Finally, we could not find any association between seminal leucocytes and sperm deformability (Fig. 3B) Seminal plasma MDA has a negative correlation with plasmatic total antioxidant capacity $r_{spearman}$ =-0.753; p=0,000. (Fig.4A). Nine patients have a value of MDA under the cut-off calculated in control group (MDA mean ± 2 SD= 0.77 m mol H_2O_2/l) with a highest value of ORAC. Using ROC test we have calculated ORAC value cut-off 1.035 mmol AA equiv/l predicting MDA production on semen with a 100% of sensitivity and 94.4% of specificity (AUC=0.765, p=0.000) (Fig.4B)

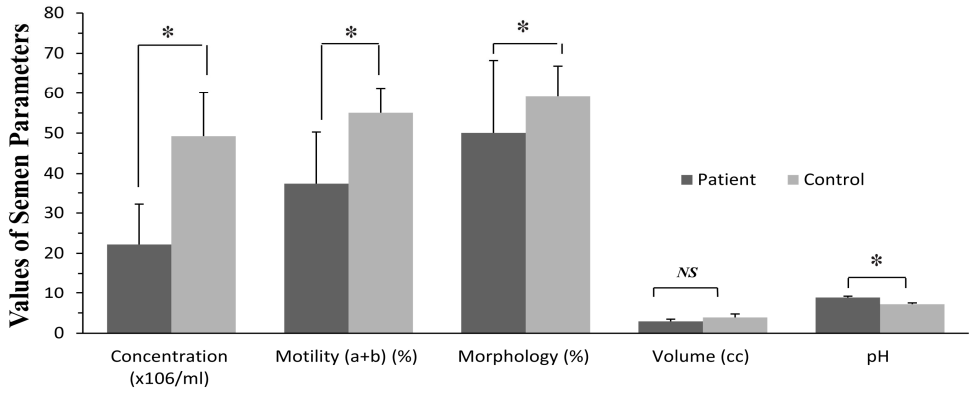

Fig.1 Semen characteristics of idiopathic infertile patient and control groups
*P; Student's t-test for independent samples. NS: no significant; * p<0.05*

Table 2. Comparison of semen parameters

Semen Parameters	Patient	Control	*p*
Concentration (x106/ml)	22.19±10.0	49.26±10.86	0.005
Motility (a+b) (%)	37.53±12.76	55.06±6.25	0.009
Morphology (%)	50.05±18.15	59.16±7.65	0.023
Volume (cc)	2.93±0.51	3.90±0.87	0.015
pH	8.83±0.35	7.15±0.32	0.11

DISCUSSION

In 2014, the American army has emphasized that infertility among male veterans serving during *Operation Enduring Freedom* and *Operation Iraqi Freedom* was about 13.8%, which is more frequent than the military

condition Post-traumatic Stress Disorder (PTSD) that was by 13.5% [47, 48].Needless to reminder that inflammatory conditions, extreme physical exercises and thermal stress (cold and heat stresses) have been reported as major sources of systemic OS in [49, 50]. However, the **e**nvironmental temperature variations are the most common stresses experienced by wide range of organisms inducing suboptimal semen quality [51].

We have selected 28 soldiers working in the Algerian Sahara during a minimum of two years for a purpose to test our null hypothesis (H_0) 'the absence of any OS impact in their poor quality of sperm', and if so, evaluate the capability of surviving and adapting to severe systemic physiological stresses by the compensatory system of Lcn-2 as an adaptive response to ameliorate the injuries induced by thermal stresses, and re-establishment of homeostasis, as described by *M.H. Roudkenar et al*., [25].

As expected, the semen parameters of infertile men (sperm count, motility, viability & morphology) were significantly lower in infertile patients than control fertile subjects. The main antioxidant defense in seminal plasma includes superoxide dismutase (SOD), catalase (CAT), Glutathione peroxidase (GPx), Vit-C, Vit.-E and Zn [52]. These molecules represent total antioxidant capacity (TAC) [53]. The major pro-oxidant reaction which may interact with DNA is the lipids chain reaction that leads to MDA formation. Many antioxidant and oxidant molecules cannot be individually measured, and such measurement is very expensive; so TAC and TP are more useful for evaluation of oxidative status [54, 55]. Thus, oxidative balnce may be assessed by ORAC , which is the most reliable marker in evaluating TAC by using hydroxyl radicals rather than peroxyl, and MDA that is very useful to assess total peroxide lipid (TP).

In the current study, we found high levels of MDA in infertile men as compared to fertile but did not correlate with sperm count and sperm motility and morphology which concords with the work of *Nabil H. et al* reported elevated seminal MDA concentration in patients with oligozoospermic and azoospermic groups [56] and *Suleiman et al* conclusion that MDA concentration in the seminal plasma was not related with the sperm concentration and motility [57]. However, our data are in contrary to others studies that shown that sperm concentration was negatively associated with ROS,[37-39] and that morphologically abnormal sperm are commonly found with reduced antioxidant capacity [16, 52, 58-60].

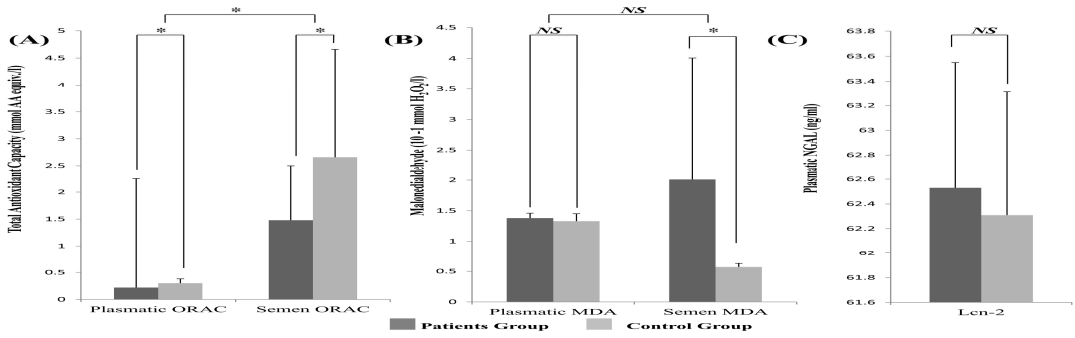

Fig.2 TAC and MDA values of semen and blood plasma in idiopathic patient and control groups show the statistically significant differences among infertile and fertile men

*p_1, Student's t-test for independent samples. p_2, Student's t-test for paired samples.NA: not available. NS: no significant;*p<0.05.*

Table 3. Comparison between blood and sperm oxidative balances

Parameter		Seminal plasma	Blood plasma	p_1
Total Antioidant Capacity (mmol AA equiv./l)	Patients	1.48±1.33	0.22±0.02	0.003
	Controls	2.66±1.02	0.3±0.04	0.009
	p_2	0.003	0.505	
Plasmatic NGAL (ng/ml)	Patients	Assay not adapted	62.53±1.02	NA
	Controls	Assay not adapted	62.31±0.9	NA
	p_2	NA	0.098	
Malonedialdehyde (mmol H2O2/l)	Patients	2.01±0.03	1.38±0.08	0.020
	Controls	0.57±0.06	1.07±0.63	0.003
	p_2	0.000	0.411	

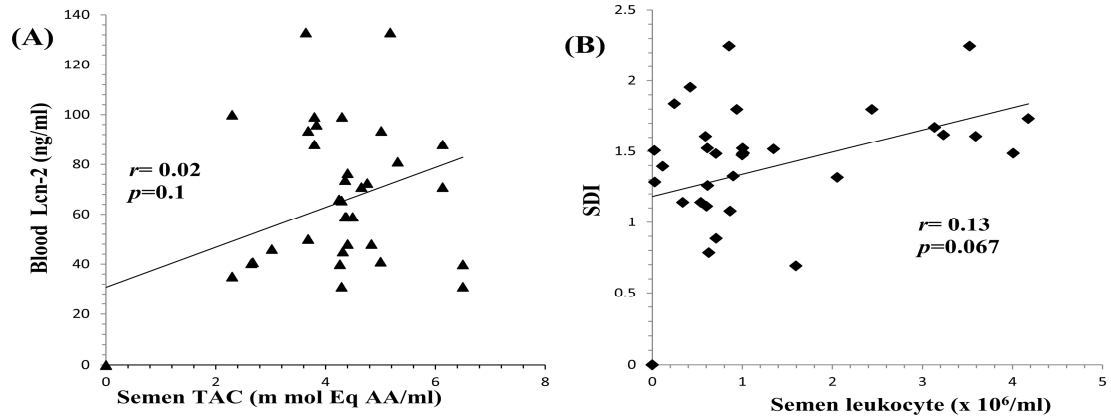

Fig.3 (A) Correlation between NGAL and plasmatic ORAC. (B) Correlation between seminal leucocytes and sperm deformability

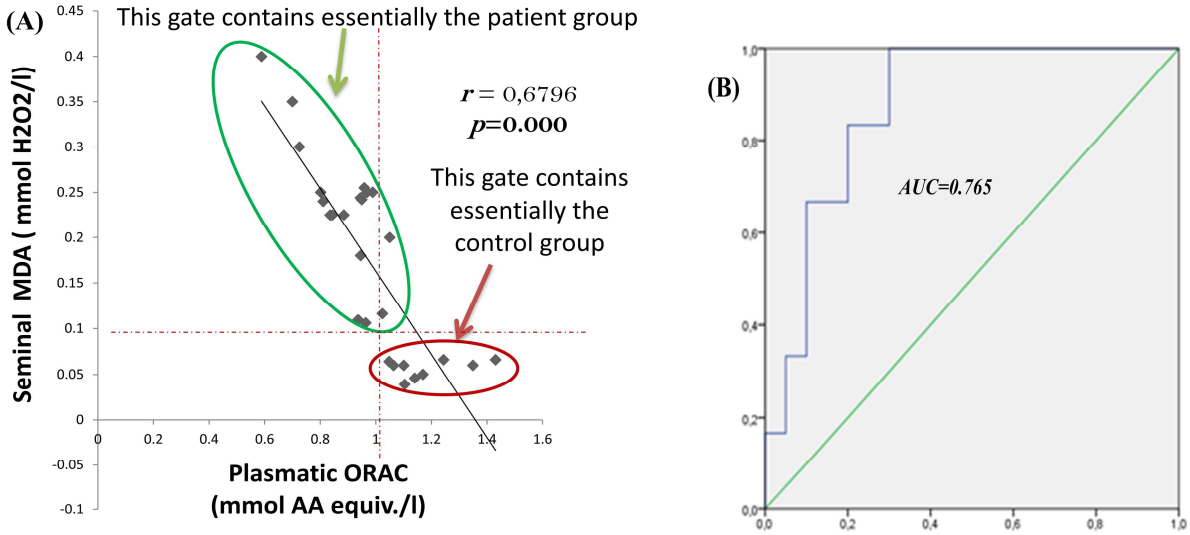

Fig. 4 (A) Correlation between seminal MDA and plasmatic TAC in infertile men. (B) Receiver operating curve for determination of an ORAC value predicting semen MDA

However, a recent study reported that high levels of ROS may be considered as an independent marker for male factor infertility, regardless to normal or abnormal semen parameters [61]. Of note, we have not found any correlation between sperm deformability and leucocytes counts. Obviously, these findings suggest that OS is not associated with impaired sperm parameters assessed by the usual WHO criteria because OS can compromise sperm fertilizing capacity without affecting seminogram [62,63]. We should notice that conflicting studies about the evidence whether high ROS levels are a cause or consequence of abnormal semen parameters and sperm damage, make it difficult to establish the clinical relevance of ROS measurement in medical practice [64, 65].

We found significantly lower seminal TAC activity in infertile men compared to fertile men which is in concordance with several studies of *Pasqualotto et al*. who had shown that control subjects had seminal TAC values at least 1.2 fold higher than that found in infertile males [66-67]. *Khosrowbeygi A et al*, *Koca et al*. and *Sharma et al*. have reported that TAC levels significantly lower in the astheno-zoospermic, astheno-terato-zoospermic and oligo-astheno-terato-zoospermic versus Control group [68-70]. Thus, low levels of TAC indicate that antioxidants are utilized to detoxify the excessive amount of ROS.

Moreover, it has been reported that NGAL acts as a cytoprotective factor against cellular stresses such as H_2O_2, the initiator of seminal lipid peroxidation chain [70, 71]. The lack of available assay for seminal NGAL forced us to

study only the plasmatic one. It was found to be lower in control group than the infertile men but, without any correlation with seminal MDA. We have to recognize that this finding, as well as several other controversies concerning OS and leucocytospermia [26] cannot be explained under the light of the actual state of knowledge.

Obviously, OS limits the fertilizing potential of these cells as a result of collateral damage to proteins and lipids in the sperm plasma membrane [64-72] by inducing chemical reactions that create small molecular mass electrophilic lipid aldehydes such as MDA that itself culminate and trigger further mitochondrial ROS generation [73]. The measure of semen MDA level seems to be a useful biomarker for sperm oxidative damage. The current data provides evidence that decreased levels of plasmatic TAC is associated with high semen toxicity by MDA, thus it may be of valuable help in monitoring semen toxicity without semen sampling, only by a blood sample.

NGAL might not be considered as a protective factor within idiopathic infertility. Generally, laboratory sperm parameters are in evolution according to WHO criteria and we notice that they may be not adapted to study semen OS. If our data can be confirmed in a larger sample of male infertility subjects, it would have been enhanced the evidence supporting the introduction of OS biomarkers in infertility routine checkup.

CONCLUSION

Ours findings suggest that oxidative status should be used as a diagnostic tool in infertile men especially in cases of idiopathic infertility and that the reference values of ROS and MDA in neat semen can be used to define the pathologic levels in infertile men and may guide in better therapeutic interventions. We suggest that the two biomarkers; seminal MDA and TAC, may be of high usefulness as independent parameters of male factor infertility, irrespective of whether these patients have normal or abnormal seminogram. On other hand, serum Lcn-2 could not be considered as a diagnostic biomarker for male idiopathic infertility at least in heat stress related infertility. Nevertheless several series of clinical trials are needed to confirm this prospect. As far as we know, our study is the first that propose a blood plasma biomarker to monitor semen quality.

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