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Effect of abamectin exposure on semen parameters indicative of reduced sperm maturity: a study on farmworkers in Antalya (Turkey)

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Summary

Environmental exposure to pesticides may cause serious health risks including fertility and reproductive function. The aim of this study was to highlight whether there is a relationship between exposure to abamectin and male fertility parameters of farmworkers. Twenty male farmworkers who were using abamectin and 20 men not exposed to pesticides were recruited as experimental and control groups, respectively. Semen analysis, molecular markers of sperm maturity and serum reproductive hormone levels were evaluated. In experimental group, high plasma abamectin levels were detected. These men have decreased sperm motility. Moreover, diminished molecular markers of sperm maturity, such as decreased hyaluronic acid (HA) binding of sperm, increased numbers of aniline blue positive sperm and increased percentage of creatine kinase (CK) positive sperm, were observed in abamectin-exposed men. Their serum testosterone, LH and FSH levels did not change significantly. We conclude that exposure to abamectin may impair male fertility by effecting semen quality.

Introduction

Farmworkers are exposed to pesticides when handling and applying pesticides and while working in fields to which pesticides have been applied, even after the re-entry interval (the period of time following a pesticide application during which workers must not enter the treatment area without wearing protective clothing and personal protective equipment) has expired, and this occupational pesticide exposure is considered an important health risk for farmworkers (Villarejo, 2003; McCauley *et al.*, 2006; Salvatore *et al.*, 2008; Bradman *et al.*, 2009). Although little research documents farmworker pesticide exposure, it has been reported that farmworkers are exposed to high levels of pesticides (Fenske *et al.*, 2003; Quandt *et al.*, 2006; Calvert *et al.*, 2008).

Abamectin is a macrocyclic lactone product derived from the soil microorganism streptomyces avermitilis and used as an agricultural pesticide worldwide. Antalya has intense agricultural pesticide use, including abamectin (ABM). It is a mixture of avermectins containing about 80% avermectin B1a and 20% avermectin B1b (Burg *et al.*, 1979; Fisher & Mrozik, 1989). These two components, B1a and B1b, have similar biological and toxicological properties (Lankas & Gordon, 1989). ABM is used as an insecticide and acaricide in many parts of the world. ABM undergoes little metabolism within the target organism, and most of the dose given to the animal is hence excreted as parent compound, primarily in the faeces with <2% in urine (Gruber *et al.*, 1990). They are highly lipophilic substances and dissolve in most organic solvents, but are poorly soluble in water (Roth *et al.*, 1993). In the environment, ABM is quickly degraded (half-life 4–21 h) by oxidative and photo-oxidative mechanisms when exposed to light in water or as a thin film on biological surfaces (e.g. leaves) or when it is bound to the soil particles and then exposed on glass plates (Wislocki *et al.*, 1989; Halley *et al.*, 1993).

Although pesticides may be valuable in agriculture, it may be highly toxic to mammals (Moline *et al.*, 2000). One of the most important toxic effects of these chemicals on living organisms is on reproductive systems (Colborn *et al.*, 1993; Toppari *et al.*, 1996; Skakkebaek *et al.*, 2001). Over the past few decades, a remarkable drop in fertility rates of men has been noticed all over the world, including both developed and developing countries (Skakkebaek *et al.*, 2006). In recent years, reproductive toxicity has been a topic of increasing interest and concern, as human exposure to a considerable number of potential toxicants is unavoidable due to contamination of air, water, ground, food, beverages, drugs and household items (Klinefelter *et al.*, 2002; Pasqualotto *et al.*, 2004). Previous reports have indicated a strong link between male infertility and exposure to more than 50 pesticides (Cox, 1996; Manfo *et al.*, 2012; Victor-Costa *et al.*, 2010; Tiwari *et al.*, 2011). Experimental studies have shown that one of these pesticides, ABM, has an important effect on male fertility parameters in animals (Elbetieha and Da'as, 2003; Xu *et al.*, 2005). Despite the large amounts of research on the various toxic effects of ABM in animals (Hsu *et al.*, 2001; Delgado & Paumgartten, 2004; Soyuncu *et al.*, 2007; Sun *et al.*, 2010), levels of ABM in male farmworkers exposed to this pesticide remain unclear. Moreover, there are limited numbers of studies evaluating its effect on human fertility. Therefore, the aim of this study was to determine the levels of ABM in farmworkers exposed to this pesticide and investigate whether there is a relationship between male infertility and exposure to ABM, a common pesticide being used by farmworkers in Antalya region of Turkey.

Materials and methods

Subject selection, data collection and semen analysis

The study was carried out in Antalya region of Turkey between May 2007 and June 2008. Forty subjects (20 controls and 20 farmworkers) agreed to participate in our study. Informed consent was obtained from each participant prior to the study. The volunteers were given an option to withdraw from the study at any time. Male farmworkers (25–39 years old, $n = 20$) who were using ABM pesticide longer than 5 years at least for 4–5 times per year were recruited as experimental group. According to their self-reported information, their experience with respect to exposure to other pesticides was unremarkable. On the other hand, peoples recruited in Antalya, considered as a control or nonexposed group (26–42 years old, $n = 20$), are men whose activities do not involve pesticide use. A detailed interview was completed with volunteers for data collection. The questionnaire was designed after assessing PubMed articles about occupational and nonoccupational risk factors well known to be responsible for semen impairment or male infertility. The questionnaire included items on age, current health status, lifestyle factors (smoking habits, coffee and alcohol consumption) and recent risk factors for pesticide exposure, including workplace activities and behaviours, and work environment. Each participant filled out a questionnaire asking if they had 'ever' or 'never' used pesticides, how those pesticides were applied, how often they used pesticides, and whether any protective equipment had been used. Blood and semen samples were collected in all subjects. We timed our collection of blood and semen samples to coincide with the middle of the growing season and the time that pesticide spraying applications were being applied to crops in the Antalya region. There was no evidence of a systemic or specific illness that might cause infertility in all subjects, according to their medical history, physical examination and laboratory tests. These laboratory tests included the determination of testosterone (T), LH and FSH. Semen of volunteers was collected by masturbation into a sterile wide mouth glass container after at least 3 days of sexual abstinence. All the assessments were performed after liquefaction of the semen. Sperm motility and concentration were assessed according to World Health Organization (WHO) criteria 2010 (World Health Organization, 2010). The proposed study was approved by the Institutional Ethical Committee of Akdeniz University with approval number 294.

Abamectin concentrations in human plasma samples were determined according to the plasma sample preparation procedure that was explained above.

Assessment of abamectin levels in plasma

Materials

Acetonitrile (ACN), methanol, triethylamine (TEA), N-methylimidazole (NMI) and trifluoroacetic anhydride (TFAA; analytical grade) were supplied from Merck (Darmstadt, Germany). ABM and doramectin (Sigma-Aldrich, analytical standard, St. Louis, MO, USA) were used as standard reference materials.

HPLC conditions

The HPLC system (Shimadzu, Columbia, MD, USA) consisted of a LC10AD VP pump and a Shimadzu RF-10AXL fluorescence detector (excitation wavelength 365 nm; emission wavelength 470 nm). The separation was carried out on a Phenomenex Luna 3 μm C18(2) column (150 × 4.6 mm i.d.; 3 μm particle size) with a Phenomenex pre-column C18 (ODS, Octadecyl; 4.0 × 3.0 mm i.d.; 5 μm particle size, Torrance, CA, USA). The mobile

phase consisting of methanol-ACN-water (95 + 3 + 2, v/v/v) was pumped at 1.1 ml min^{-1}, and 20 μl of sample was injected into the HPLC system.

Plasma sample preparation procedure

One millilitre of ACN:water (4 + 1, v/v) was added to each plasma sample (1 ml) and vortexed for 5 min. The samples were centrifuged at room temperature for 5 min at 2000 ***g***. After centrifugation, the supernatant was taken and transferred to a reservoir connected to a Bakerbond Octyl (C8) cartridge. The cartridge was previously activated with 5 ml of methanol and conditioned with 5 ml of water. After applying the sample extract, the cartridge was washed with 2 ml of water followed by 1 ml water:methanol (3 + 1, v/v). The analyte was eluted with 1.2 ml of methanol, collected in a polypropylene test tube and evaporated to dryness under nitrogen at 60 °C. Plasma were then derivatised with 100 μl of NMI in ACN (1 + 1, v/v) and 150 μl of TFAA in ACN (1 + 1, v/v). An 20-μl aliquot of the sample was then injected into the HPLC system.

For quantification purposes, calibration curves for ABM were prepared. The addition of 20 μl of the standard working solutions resulted in calibration curves with ABM concentrations of 1–100 ng ml^{-1} plasma. The curves were linear over this range ($r = 0.998$). The detection limit (LOD) and the limit of quantitation (LOQ) were defined as three and ten standard deviation plus mean blank value, respectively. LOD and LOQ values were calculated as 0.05 and 0.12 ng ml^{-1} for ABM, respectively.

Assessment of serum hormone levels

ELISA was used to estimate the levels of serum T, LH and FSH.

Hyaluronic acid-binding assay (HBA)

Commercial HBA kits were purchased from Biocoat (Fort Washington, PA, USA). HBA test was performed following the manufacturer's instructions. Briefly, 10 μl of semen was added to the centre of the HBA chamber, and the Cell-Vu grid cover slip was put on without entrapping air bubbles. After incubation of the slide for 15 min, the percentage of hyaluronan-binding sperm was calculated using the bound motile sperm divided by the sum of bound and unbound motile sperm counted in the same squares and then multiplied by 100.

CK-B immunocytochemistry

Initial semen were fixed with 3.7% paraformaldehyde for 20 min. The spermatozoa were exposed to a 3% bovine serum albumin blocking solution. The sperm were overlaid with a 1 : 1000 dilution of polyclonal anti-CK-B antiserum (Chemicon Co, Temecula, CA, USA). The slides were treated with a biotinylated second antibody at a 1 : 1000 dilution and were exposed to a Vector horseradish peroxidase/ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. The avidin-biotin-complex (ABC)-treated slides were further processed with diaminobenzidine (DAB; Sigma, St. Louis, MO, USA). The developed Brown colour highlighted sperm with various degrees of cytoplasmic retention.

Aniline blue staining of sperm chromatin

Sperm smears were dried on glass slides and stained with a 5% aniline blue solution acidified to approximately pH 3.5 with acetic acid. The slides were washed and air dried, and a coverslip was applied. Because of persistent histones, sperm with immature chromatin were stained to various intensities of blue (Hammadeh *et al.*, 1996; Huszar *et al.*, 2003).

FISH method

All steps, including the preparation of sperm nuclei and the processes of FISH, were carried out essentially as described by Kovanci *et al.* (2001). Three-colour FISH was performed using centrometric probes for the X,Y and 17 chromosomes (Vysis, Downers Grove, IL, USA). Sperm nuclei were scored according to published criteria (Martin & Rademaker, 1995). Scoring was performed on an Olympus BX61 epifluorescence microscope.

Statistical analysis

Data are presented as the mean ± SEM and were analysed using the GRAPHPAD Prism™ software version 3.0 (San Diego, CA, USA). Significant findings were further compared by analysis of variance (one-way ANOVA) with Tukey's *post hoc* test. The frequencies were compared among groups using the Fisher's exact test. Levels of significance were set at $P < 0.05$.

Results

As shown in Table 1, general characteristics of study subjects were not significantly different between the two groups; therefore, it was not controlled in subsequent adjustments. The consumption of alcohol, tobacco, as well as smoking habits, was similar in control and ABM-exposed group. Similarly, there were no significant differences in the frequencies of general and reproductive

Table 1 General characteristics of study subjects

Characteristics	Frequency (%)	
	Control (n = 20)	Abamectin-exposed (n = 20)
Tobacco smoking		
None	70	65
Everyday	15	20
Sometimes	15	15
Frequency of alcohol intake		
None	55	60
Everyday	0	0
Sometimes	45	40
Coffee/tea intake		
None	15	10
Everyday	80	75
Sometimes	5	15
Reported disorders		
Cardiovascular diseases	0	0
Diabetes/obesity	10	5
Reproductive disorders/dysfunction	0	0
Other diseases	0	0

Frequencies were compared to control group using Fisher's exact test.

health diseases/dysfunction between control group and ABM-exposed group (Table 1).

Abamectin levels

The peak retention time of ABM in plasma was 6.7 min. Plasma ABM levels were found to be significantly higher in farmworkers when compared with control group (Fig. 1).

Serum reproductive hormone levels

Testosterone, LH and FSH levels were within the normal ranges in ABM-exposed group (Table 2).

Semen parameters and molecular markers of sperm maturity

As shown in Table 2, there were no significant differences in mean sperm concentrations between control group and ABM-exposed experimental group. However, lower sperm motility and higher semen volume were determined in farmworkers that were exposed to this pesticide.

Molecular markers of sperm maturity data are summarised in Table 3. Sperm cellular maturity and cytoplasmic retention can be detected by CK-B immunocytochemistry. Observation of the stained slides can reveal proportions of clear-headed (mature, intermediate and darkly stained immature, with substantial cytoplasmic retention or heavily fragmented DNA) sperm in semen smears. There was a significant decrease in clear-headed

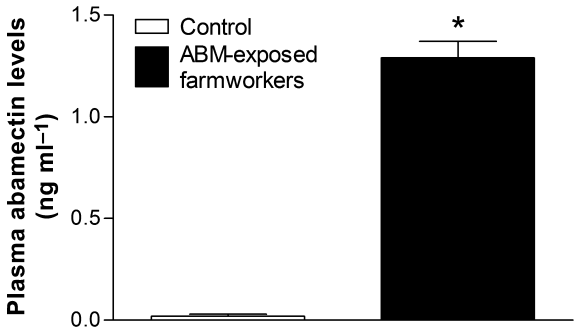

Fig. 1 Plasma levels of abamectin in control men and abamectin (ABM)-exposed farmworkers. *P < 0.0001 compared with controls.

Table 2 Reproductive hormone levels (ng ml^{-1}) and semen parameters in control and abamectin-exposed farmworkers

	Control	Abamectin-exposed
LH [normal range (1.70–8.60)]	3.33 ± 0.38	5.02 ± 0.68
FSH [normal range (1.50–12.40)]	3.05 ± 0.39	4.30 ± 0.65
Testosterone [normal range (2.80–8.00]	5.84 ± 0.95	5.17 ± 0.45
Sperm concentration (M ml^{-1})	68.2 ± 12.7	64.9 ± 13.7
Sperm motility (%)	66.2 ± 2.70 (52–77)	42.1 ± 7.30* (0–75)
Semen volume (ml)	2.30 ± 0.50	4.10 ± 0.50*

Data are presented as (mean ± SEM).
*P < 0.05 compared to controls. Parentheses indicate minimum and maximum values of sperm motility.

sperm in semen of ABM-exposed males when compared with controls.

In semen of ABM-exposed farmworkers, there is a higher proportion of sperm with increased aniline blue staining, which highlights the presence of persistent histones in chromatin. Thus, to ascertain nuclear maturity of single spermatozoa, we prepared aniline blue-stained slides. Percentage of sperm with normal chromatin decreased significantly in farmworkers exposed to ABM.

Hyaluronic acid-binding assay test is based on previous reports of hyaluronic acid selectively binding to mature sperm with intact acrosome and better morphology (Huszar *et al.*, 2003). There was a statistical significant difference in HBA scores between control and ABM-exposed men. HBA scores of ABM-exposed farmworkers were lower than HBA scores of control group.

Evaluation of chromosomal aneuploidies by FISH

No significant differences were observed between control and ABM-exposed men for numerical chromosomal

Table 3 Molecular markers of sperm maturity in control and abamectin-exposed farmworkers

	Control	Abamectin-exposed
CK-B (%)	88.6 ± 1.80 (75–96)	79.8 ± 3.00* (62–94)
Aniline Blue (%)	84.1 ± 2.40 (71–95)	76.5 ± 2.70* (57–93)
Sperm hyaluronic acid-binding assay (%)	83.0 ± 2.60 (66–91)	51.2 ± 6.80* (15–77)

Data are presented as (mean ± SEM).
*$P < 0.05$ compared to controls. CK-B (creatine kinase-brain type) negative or aniline blue negative cells represent light sperm, which are not stained with either of these markers. Percentages for CK-B and aniline blue indicate light sperm with normal maturity. Parentheses indicate minimum and maximum values of molecular markers of sperm maturity.

Table 4 Sperm aneuploidy rates in control and abamectin-exposed farmworkers

	Control	Abamectin-exposed
Number of cells evaluated	1305 ± 88.5	1232 ± 55.3
Sex chromosome disomy (%)	2.32 ± 0.25	2.25 ± 0.38
Disomy 17 (%)	1.47 ± 0.17	1.37 ± 0.28
Diploidy (%)	0.66 ± 0.10	0.94 ± 0.13

Data are presented as (mean ± SEM).

abnormalities. Rate of sperm aneuploidies from farmworkers exposed to ABM was within the normal ranges (Table 4).

Discussion

Although a number of pesticides have been shown to induce impairment of spermatogenesis in humans (Swan *et al.*, 2003; Meeker *et al.*, 2008), there are limited number of studies evaluating effects of ABM on farmworkers. The goals of this paper have been to analysis of plasma ABM levels in male farmworkers and investigate the possible detrimental effects of ABM on male fertility parameters. To the best of our knowledge, our study is the first one that examines the effects of ABM exposure on semen parameters in male farmworkers. ABM pesticide was chosen in this study, because it is widely used by farmworkers in many parts of the world, including Turkey.

Most farmworkers are exposed to ABM pesticide across an agricultural season; however, the levels of this pesticide in these farmworkers are not known in detail. Results of the detailed interview indicated that the pesticide was sprayed with minor or no protective equipment. As a consequence of this inadequacy of protective equipment, they were exposed to ABM that might enter their organism by absorption after contact with the skin or eyes (dermal), or by breathing into the lungs (inhalation) (Fenske & Elkner, 1990; Lu, 2007). The results of this study have shown that blood samples collected from the farmworkers exposed to ABM contained high levels of ABM. When the concentrations of ABM were compared between control men and farmworkers, statistical significance was obtained. A series of previous studies have reported that environmental factors had the primary role causing the observed adverse trends in the male reproductive health problems. One of them, ABM pesticide, may effect reproductive systems of male farmworkers. A possible relationship between ABM exposure and male infertility has been investigated in limited animal studies. These studies have shown that ABM has an important effect in the motility of sperm of rosy barb (*Puntius conchonius*) because this pesticide attacks the midpiece of sperm, and the mitochondrias, modifying the motility (Xu *et al.*, 2005). Elbetieha & Da'as (2003) have also indicated that exposure to the pesticide ABM at the 1.87 or 2.13 mg per animal per day doses for 6 weeks would have adverse effects on fertility and reproduction in adult male rats. In accordance with these results, we have found that ABM may be toxic to the reproductive system of male rats (Celik-Ozenci *et al.*, 2011). In this study, we have shown that pregnancy rates decreased in unexposed female rats after mating with ABM-exposed males. The low fertility rates in ABM-exposed rats indicate that ABM may reduce the fertility of these male rats. Although levels of ABM in ABM-exposed men were lower than the ABM-exposed rats, some of these men had considerably high levels of ABM (11.8 ng ml^{-1}) similar to the levels of rats. On the other hand, long-term repeated exposure to this pesticide may amplify the detrimental effect of ABM on male semen parameters. Moreover, sperm motility changes in male rats exposed to ABM were observed. Taking the above into account, it may be suggested that ABM also causes harmful effects on male fertility of farmworkers if they do not follow the recommended safety practices.

In this study, although there were no significant differences for numerical chromosomal abnormalities between two groups, poor semen characteristics including decreased sperm motility and increased abnormality of molecular markers of sperm maturity were observed in ABM-exposed farmworkers when compared with controls. It has been suggested that studies directed to male infertility or to reproductive toxicity because of occupational or environmental factors could be improved if validated biomarkers of sperm maturation and function were also utilised in addition to the conventional sperm concentration and motility parameters (Huszar *et al.*, 2004). Therefore, in this study, objective markers of sperm maturity have also been analysed in addition to the conventional semen analysis. It is generally accepted that semen

parameters alone do not directly reflect the fertility status of a male. On the other hand, it has been suggested that reduction in molecular markers of male fertility is likely to be important from the perspective of reproductive toxicity assessments, because testicular function of exposed men might be on the decline with respect to sperm maturation, while their sperm concentrations are still maintained at WHO normozoospermic levels (Huszar *et al.*, 2004). In the selection of sperm attributes for this toxicology study, we focused on conventional semen parameters and on the objective biochemical markers of sperm maturity and function that have previously been identified. Hyaluronic acid-binding scores of ABM-exposed infertile farmworkers decreased significantly when compared with nonexposed men. Moreover, semen from these men had a higher ratio of sperm with cytoplasmic retention and persistent histones, indicating sperm immaturity due to insufficient cytoplasmic retention and chromatin remodelling. For the first such marker, sperm CK, immunocytochemical studies indicated that high sperm CK activity was related to increased CK concentrations, as well as to abnormal and amorphous sperm head size and shape (Huszar *et al.*, 1988a,b; Huszar & Vigue, 1993). These findings suggested that a sperm developmental defect may occur in the last phase of spermiogenesis, the so-called cytoplasmic extrusion phase, when the cytoplasm (unnecessary for the mature sperm) is normally left in the adluminal area as 'residual bodies'. Huszar *et al.* (1990) examined the utility of the CK activity marker in couples with oligospermic husbands whose wives were treated with intrauterine insemination. The results showed that increased CK activity was related to failure to cause pregnancies, whereas the sperm concentrations provided no predictive power. Moreover, it has been suggested that CK may be a sensitive indicator of sperm quality and maturity in the follow-up of patients treated for male factor infertility (Hallak *et al.*, 2001). In our study, the percentage of sperm without cytoplasmic retention (light CK-B sperm) decreased significantly in ABM-exposed men indicating a sperm developmental defect during cytoplasmic extrusion. Aniline blue staining detects the presence of histones and, therefore, indirectly infers the presence of lower amounts of protamines in the sperm nucleus (Hofmann & Hilscher, 1991). Franken *et al.* (1999) showed that there was significantly higher percentage of aniline blue positive spermatozoa among men with teratozoospermia when compared with normozoospermic (51% versus 26%). Despite normal semen analysis in Franken's study, the mean percentage of positive aniline blue staining (dark sperm) was 31.6% in the subfertile group and 14.1% in the control group. In our study, the mean percentage of negative aniline blue staining (light sperm) was 76.5% in ABM-exposed men and 84.1% in the control group indicating that sperm chromatin remodelling was diminished in ABM-exposed men. It is also important to remember that sperm that are able to bind to HA are mature and have completed the spermiogenetic processes of sperm plasma membrane remodelling (indicated by HBA scores) (Huszar *et al.*, 2003). Recently, a study by Parmegiani *et al.* (2010) showed that injection of HA-bound spermatozoa (HA-ICSI) significantly improved embryo quality and implantation because HA favours selection of spermatozoa without DNA fragmentation and with normal nucleus, resulting in improvement of embryo quality. Accordingly, the results of our study have shown that sperm HBA scores are significantly lower in ABM-exposed men, which may indicate their poor fertility potential. Because the general characteristics of study subjects were not significantly different between the two groups, observed detrimental effects on sperm motility and molecular markers of sperm maturity in farmworkers may be related with high plasma ABM levels in ABM users.

In conclusion, the results of this study are not only important as they provide baseline data on the concentration of ABM pesticide in Turkish farmworkers but are also very important as the first evaluation of the effects of ABM pesticide on reproductive systems of farmworkers. Our results have clearly shown that farmworkers who were exposed to ABM had increased plasma ABM levels and impaired sperm dynamics. It is recommended that exposure to ABM should be limited and highly regulated due to its potential hazardous effects on male fertility.

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