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Ameliorative Effects of Curcumin on the Spermatozoon Tail Length, Count, Motility and Testosterone Serum Level in Metronidazole-treated Mice

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Abstract: Metronidazole (MTZ) is used as an antiparasitic drug. Curcumin is considered as anti-oxidant and anti-inflammatory agent. The ameliorative effects of curcumin on MTZ induced toxicity on mice spermatozoon tail length, count, motility and testosterone level were investigated. MTZ was administered in 500 and 165 (high and therapeutic doses) mg/kg/day, with and without curcumin (100 mg/kg/day). After 16 days the above parameters were assessed. Spermatozoon count and motility and serum testosterone level MTZ-treated (500 and 165) mice were reduced. In the mice treated with MTZ+curcumin these parameters decreased but in a lesser extent than the MTZ-treated animals. Mid-piece and total lengths of the spermatozoon tail in control animals were 31.6±9.0 μm and 100.3±15.0 μm and in the mice treated with high doses (500) of MTZ were reduced. The mid-piece and total spermatozoon tail length has been decreased in a lesser extent in the mice treated with high dose MTZ+curcumin than the mice treated with high dose MTZ ($p<0.01$). But the length was not changed in animals treated with therapeutic dose of MTZ. It means curcumin treated animals had ~52% and ~39% average increase in mid-piece and total lengths in comparison with the MTZ-treated (500) animals. Stereological estimation of the sperm tail length, including sampling of spermatozoa and also counting of the intersections of their tails with the stereological grids was a rapid technique and took only 5–10 minutes. It can be concluded that curcumin has an ameliorative effect on the spermatozoon, testosterone level and tail length in MTZ-treated mice.

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Introduction

Spermatozoa morphology has been frequently used as indicator of toxicity and mutagenicity in mammals (Hemavathi and Rahiman, 1993; Khan and Sinha, 1996; Bustos-Obregón and González-Hormázabal, 2003; Joshi et al., 2003). The new parameters as mid-piece and total spermatozoa tail length were suggested here for better qualifying of the spermatozoa quantitative morphology. Thus the effects of MTZ and curcumin on the spermatozoa analysis and tail length were studied here.

MTZ is used as a wide spectrum biocide and antiparasitic drug in the treatment of *Entamoeba histolitca*, *Giardia lamblia*, *Trichomonas vaginalis* and anaerobic organisms in general. Genotoxic activity of this drug can induce alterations in somatic and germinal cells (McClain et al., 1989; Grover et al., 2001). It has been reported that after 6 weeks of treatment of rats with MTZ (400 mg/kg/day), decreases of testicular weight, testicular and epididymal spermatozoon counts and abnormal spermatozoon morphology with degeneration of seminiferous tubules can be seen (McClain et al., 1989; Grover et al., 2001). Abnormalities in the flagellum or the head have been reported in the morphological analysis after treatment with MTZ (El-Nahas and El-Ashmawy, 2004; Mudry et al., 2007). Metronidazole also induced a significant decrease in the number of motile spermatozoon and an increase in the abnormal ones (El-Nahas and El-Ashmawy, 2004; Mudry et al., 2007). The tail of a spermatozoon is one of the important factors determining the propulsive velocity of the cell and hence it's potential to achieve fertilization. Spermatozoon motility is mainly related to the energetic component that is the mid-piece and length of the spermatozoon's tail, being the kinetic component of the spermatozoon. The effects of MTZ on the mid-piece and total spermatozoon tail length have received less attention. It has been reported that metronidazole acts as a testosterone biosynthesis inhibitor (McClain et al., 1989; Grover et al., 2001). Thus testosterone serum level was evaluated here.

On the other hand, the effects of curcumin on the spermatozoon parameters are researched in this study. Curcumin is the principal curcuminoid found in turmeric and is generally considered as its most active constituent (Duvoix et al., 2005; Sharma et al., 2005). Turmeric exhibits anti-tumor, anti-inflammatory and anti-infectious activities with low toxicity (Cohly et al., 1998; Farombi et al., 2007; Mathuria and Verma, 2008). In the present study, the spermatozoon length estimation by the modern rapid stereological methods without need to use any image analysis software is presented. Thus, on the basis of these findings, the present study investigated the beneficial potential of curcumin against high or therapeutic doses of MTZ induced toxicity by studying the changes in the count, motility, mid-piece and total tail length of mice spermatozoon. The beneficial potential role of curcumin alone or in the amelioration of MTZ induced changes was also investigated. A rapid stereological method of tail length estimation is presented here.

Material and Methods

Animals and treatments

BALB/c male mice weighing 35–40 g and 9 weeks age were procured from the Laboratory Animal House of Shiraz University of Medical Sciences, Shiraz, Iran. The mice were acclimatized prior to the experiment and were divided into six groups, each including 5 mice. The control group received distilled water by gavages. The second group received high doses (500 mg/kg/day) of metronidazole (Noorafshan et al., 2010). The third group received high doses (500 mg/kg/day) of metronidazole and 100 mg/kg/day curcumin and the forth one administrated therapeutic doses (165 mg/kg/day) metronidazole (Noorafshan et al., 2010). The fifth group received therapeutic doses (165 mg/kg/day) of metronidazole and 100 mg/kg/day curcumin and the sixth group was administrated 100 mg/kg/day curcumin. All the administrations were by gavages for 14 days (the duration of MTZ prescription in some medical cases) and the mice were euthanized after 30 days (nearly a full spermatogenesis period of mouse). All of the experiments were done according to the rules of the Ethics Committees of Shiraz University of Medical Sciences, Shiraz, Iran.

Sample collection and seminal analysis

The procedure for obtaining and analyzing the semen was the same as that used by Karbalay-Doust et al. (2007). The spermatozoon samples obtained from the distal region of the right vas deferens of the mice were used in the present study. This involves excision of a small piece (1.0 cm) of the vas deferens just distal to the cauda epididymis. The semen samples were placed in a Petri dish containing a buffer (Hank's Balanced Salt Solution) and shaken gently at 37 °C for 15 min. The total spermatozoon count and motility were determined through microscopic examination.

Spermatozoon count

The semen samples were spread on a hemocytometer and studied using an optical microscope. The spermatozoa heads were counted manually. 300–400 spermatozoa were counted in each mouse. The data were expressed as the total number of spermatozoa/ml.

Spermatozoon motility

Aliquots of the spermatozoon suspension prepared for analysis were placed on a slide warmed at 37 °C. The slides were evaluated in 10 microscopic fields. The fields were selected randomly and 200–300 spermatozoa per animal were analyzed at a final magnification of 1000×. The motile spermatozoon fraction was defined as the mean number of motile spermatozoon × 100/total number of the spermatozoon. The motility of each spermatozoon was graded "a", "b", "c" or "d", according to whether it showed:

"a" – rapid progressive motility,
"b" – slow or sluggish progressive motility,
"c" – non-progressive motility,
"d" – immotility.

If more than 50% of spermatozoon was graded as "c" or "d", it was considered as abnormal motility.

Stereological study

The spermatozoon samples were also spread on microscopic slides and stained with Eosin-Y. Each slide was studied using a video-microscopy system, composed of a microscope (Nikon E-200) linked to a video camera, a computer, and a monitor to determine mean spermatozoon tail length at a final magnification of 2100×. An oil immersion objective lens ×60 with numerical aperture of 1.4 was used to achieve a better recognition of the tail. The tail of spermatozoon consists of mid-piece, principal and end pieces are considered as one remaining unit. Length of the tail on microscopic slides was estimated according to the stereological rules (Rønn et al., 2000; Howard and Reed, 2005; Noorafshan and Karbalay-Doust, 2010). The microscopic fields were sampled in a systematic random manner, by

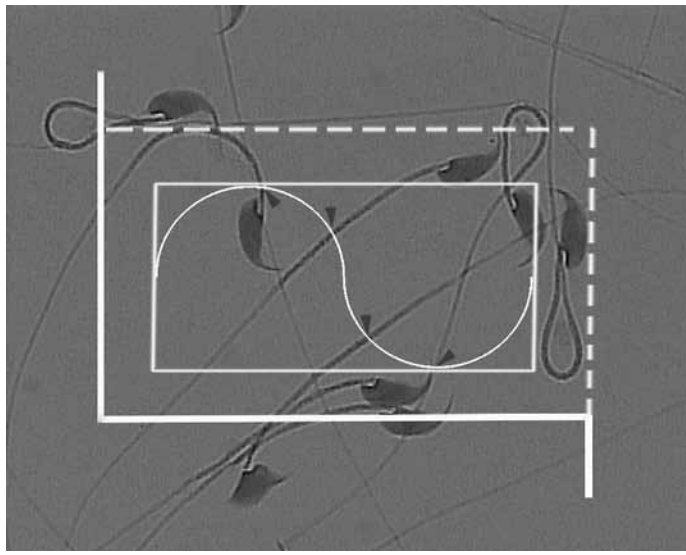

Figure 1 – A test system composed of two components is superposed on the image. The first component is an unbiased counting frame (the large frame) with inclusion (dotted) and exclusion (bold) lines. If the spermatozoon head lay inside the frame and does not touch the exclusion lines, it is sampled (here 5 spermatozoa). The second component is a basic tile (small rectangle frame) with a Merz grid inside it (two semicircles). The spermatozoon tail length is estimated using the following formula: $L = (\pi/2)\cdot(a/l)\cdot(1/asf)\cdot\Sigma I$ where: "a/l" is the Merz grid constant, "asf" is the area of the basic tile divided by the area of the counting frame and "ΣI" is the summation of the intersections of the tails with the semicircles (arrowheads; here 4). The total estimated length is divided by the number of counted spermatozoa to achieve tail length per spermatozoon.

moving the microscope stage in an equal interval along the X- and Y-direction of the microscope stage, using the stage micrometer of the microscope. 100–170 spermatozoa were sampled on each slide of the mouse. According to the stereological rules, to have an acceptable precision, at least 5 subjects in each group are sufficient for comparison and a 100–200 probe interaction (e.g. sampling of 100–200 spermatozoon's heads by counting frame, or 100–200 intersections of Merz grid with spermatozoon's tail) is sufficient (Rønn et al., 2000; Howard and Reed, 2005; Noorafshan and Karbalay-Doust, 2010). In each sample field, a test system composed of two components was superposed on the image on the monitor. The first component was the counting frame. After calculating the magnification, the counting frame size was 83.3 μm × 52.8 μm (Figure 1). If a spermatozoon's head lay inside the frame and did not touch the exclusion lines (left and inferior borders of the frame), it was sampled. Another component was a basic tile of the system with an area "X·Y" (here 53.3 μm multiplied by 26.6 μm) and a Merz grid inside it (Figure 1). The following formula was used for estimating the mean spermatozoon tail length (Rønn et al., 2000; Howard and Reed, 2005; Noorafshan and Karbalay-Doust, 2010):

ΣL (total flagella) = $(\pi/2)\cdot(a/l)\cdot(1/asf)\cdot\Sigma I$

L (tail) = $\Sigma L/\Sigma N$

where: "a/l" was the Merz grid constant which was obtained dividing the area of each basic tile (X·Y) by length of semicircles. Within this tile, there were two semicircles of length of π·d (perimeter of a circle), where "d" was the diameter of the circle and "asf" was the area sampling fraction. The "asf" was calculated by division of the area of the basic tile by the area of the counting frame. "ΣI" was the total of the intersections of the tails (mid-piece or remaining unit) with the semicircles (Figure 1). "ΣN" was the total number of the counted spermatozoa in the unbiased counting frame in all fields.

Hormone assay

One millilitre blood samples were obtained from the heart of each mice and the plasma was separated by centrifugation and stored at –20 °C for the subsequent hormone assays. T concentration was measured in plasma samples using a kit with sensitivity to T was 0.9 ng/dl.

Statistical analysis

The data were compared using Kruskal-Wallis and Mann-Whitney U-test. $p<0.05$ was considered as significant.

Results

Spermatozoon count

Spermatozoon count in high dose MTZ-treated mice was ~77% and in therapeutic doses MTZ-treated mice was ~64% lower in comparison with the control ($p<0.01$).

In animals treated with MTZ 500 plus curcumin and with MTZ 165 plus curcumin, spermatozoon count were decreased in a lesser extent in comparison with the animals receiving MTZ 500 and MTZ 165, respectively ($p<0.05$) (Table 1).

Percentage of motile spermatozoon

Percentage of the motile spermatozoon in high and therapeutic doses of MTZ-treated mice showed a significant reduction in comparison with the control ($p<0.01$). In animals treated with MTZ 500 plus curcumin and with MTZ 165 plus curcumin, the percentage of the motile spermatozoon were reduced in a lesser quantity in comparison with the animals receiving MTZ 500 and MTZ 165, respectively ($p<0.05$). It should be emphasized that the mice treated with curcumin lonely, showed a higher percentage of motile spermatozoon in comparison with the control ones (Table 1).

Spermatozoon tail length

The spermatozoon tail length was not changed in animals treated with therapeutic dose of MTZ. The mid-piece and total lengths of the spermatozoon tail in high dose MTZ-treated mice was reduced by ~39% and ~31% in comparison with control animals, respectively ($p<0.02$). The mid-piece and total spermatozoon tail length has been decreased in a lesser extent in the mice treated with high dose MTZ and curcumin than in the mice treated with high dose MTZ alone ($p<0.01$) (Table 2). It means the curcumin treated animals had ~52% and ~39% average increase in mid-piece and total lengths in comparison with the MTZ-treated (high dose) animals.

Serum levels of the testosterone

Serum testosterone level was decreased in high and therapeutic doses MTZ-treated mice by ~95% and ~90% in comparison with the control animals,

Table 1 – Mean ± standard deviation of the spermatozoa count ($\times 10^6$/ml) and percentage of motile spermatozoa in control, high (MTZ 500) or therapeutic doses (MTZ 165) metronidazole-treated with or without curcumin (CUR) groups

Groups	Count	Motile
Control	6.7 ± 0.9*	57.6 ± 8.0*
MTZ 500	1.5 ± 0.1	14.6 ± 10.6
MTZ 500+CUR	2.5 ± 1.0**	28.4 ± 9.0**
MTZ 165	2.4 ± 2.4	25.2 ± 11.0
MTZ 165+CUR	5.3 ± 1.8**	38.8 ± 4.1**
CUR	6.0 ± 1.0	76.7 ± 11.5***

*p<0.01 (Control vs. MTZ 500) or (Control vs. MTZ 165), **p<0.05 (MTZ 500+CUR) vs. (MTZ 500), (MTZ 165+CUR) vs. (MTZ 165), ***p<0.04 (CUR) vs. (Control)

Table 2 – Mean ± standard deviation of the mid-piece and total spermatozoon tail length (μm) in control, high (MTZ 500) or therapeutic doses (MTZ 165) metronidazole-treated with or without curcumin (CUR) groups

Groups	Mid-piece length	Total length
Control	31.6 ± 9.0	100.3 ± 15.0
MTZ 500	19.2 ± 6.0**	69.0 ± 6.0**
MTZ 500+CUR	29.5 ± 3.0*	95.0 ± 5.0*
MTZ 165	27.8 ± 8.0	108.9 ± 8.0
MTZ 165+CUR	27.6 ± 5.0	109.1 ± 10.0
CUR	31.9 ± 4.0	107.5 ± 11.0

*p<0.01 (MTZ 500+CUR) vs. (MTZ 500), **p<0.02 (MTZ 500) vs. (Control)

Table 3 – Mean ± standard deviation of the testosterone serum level (ng/dl) in control, high (MTZ 500) or therapeutic doses (MTZ 165) metronidazole-treated with or without curcumin (CUR) groups

Groups	Testosterone
Control	2.0 ± 1.08
MTZ 500	0.1 ± 0.10*
MTZ 500+CUR	0.4 ± 0.21**
MTZ 165	0.2 ± 0.08*
MTZ 165+CUR	0.7 ± 0.21**
CUR	1.7 ± 0.84

*p<0.001 (MTZ 500 vs. Control) or (MTZ 165 vs. Control), **p<0.01 (MTZ 500+CUR) vs. (MTZ 500), (MTZ 165+CUR) vs. (MTZ 165)

respectively (p<0.001). Testosterone level was reduced in a lesser amount in the mice treated with MTZ plus curcumin in comparison with high or therapeutic doses MTZ (p<0.01) (Table 3).

Discussion

The present study reports the ameliorative effects of curcumin on the spermatozoon count, motility, tail length in mice treated with high and therapeutic doses metronidazole. Spermatozoa morphology has been frequently used as indicator of toxicity and mutagenicity in mammals (Hemavathi and Rahiman, 1993; Khan and Sinha, 1996; Bustos-Obregón and González-Hormázabal, 2003; Joshi et al., 2003). But the new parameters as mid-piece and total spermatozoa tail length was suggested here for better qualifying of the spermatozoa quantitative morphology. There are several evidences revealing a higher incidence of abnormal karyotypes in the embryos grow from abnormal spermatozoa (Lee et al., 1996; Kishikawa et al., 1999). Thus quantifying the all spermatozoa parameters including tail length

should be important. In the present study we focused on a new parameter which alteration is not possible to detect without stereological estimation or image analysis. Reported are abnormalities in the flagellum or the head of the spermatozoon in the murines treated with MTZ and also a decrease in the percentage of the motile spermatozoon (Mudry et al., 2007). The data presented in this study are in the same line with those of above-mentioned articles. The tail of a spermatozoon is one of the important factors determining the swimming ability of the cell in achieving fertilization. The spermatozoon motility is mainly related to the energetic component that is the mid-piece and to the length of spermatozoon tail, being the kinetic component of the spermatozoon. As our data showed, the length of all parts of the spermatozoon tail is influenced by a high dose of MTZ but not the therapeutic dose. Thus, the reason for the decrease in the spermatozoon motility might be the effects of MTZ on both energetic and kinetic components of the spermatozoon tail. This observation is in a same line with El-Nahas and El-Ashmawy (2004) and Mudry et al. (2007). Despite the fact that spermatozoon motility is reduced in both high and therapeutic doses of MTZ, its morphology (tail length) is changed only in high dose of MTZ and so the effect of MTZ on spermatozoon motility is more complex. The mechanism by which MTZ induces its effects may be the changes in hormones levels or drug genotoxicity. Some authors claim that the mean serum FSH, LH and testosterone value were also lowered in treated animals and the toxic effects of MTZ were probably mediated by a decrease in the circulating hormones responsible for spermatogenesis (McClain et al., 1989; Grover et al., 2001). Interestingly, curcumin ameliorates these effects. Protective effects of curcumin on the testis has been reported in oxidative damage induced by cisplatin, sodium arsenite, aflatoxin, ischemia-reperfusion injury, di-n-butyl phthalate in laboratory animals (Farombi et al., 2007; Mathuria and Verma, 2007; El-Demerdash et al., 2009; Ilbey et al., 2009; Wei et al., 2009). Curcumin is also able to protect the Leydig cells of mice from damage induced by chronic alcohol administration (Giannessi et al., 2008). One of the possible ameliorative mechanisms of curcumin on the above-mentioned parameters is to scavenge the free radicals and thereby act as good antioxidants. The other mechanism might be increase in testosterone serum level. It has been shown that the testosterone plasma levels was increased in the alcohol plus curcumin treated mice (Giannessi et al., 2008). The stereological method presented here for estimating the tail length is a simple way without need to trace the tail in the images of spermatozoon. Rønn et al. (2000), have shown that in estimation of neurite length using the described method, the stereological estimation of the length was approximately five times less time consuming than the conventional method of neurite tracing (Rønn et al., 2000; Noorafshan and Karbalay-Doust, 2010). Counting the number of sampled sperm and also intersections of their tail with the stereological grids takes 5–10 minutes (Rønn et al., 2000; Howard and Reed, 2005; Noorafshan and Karbalay-Doust, 2010). It was shown that the method is not only faster but also

appears to be less difficult and laborious in comparison with the conventional methods (Rønn et al., 2000). There is no need to have any software to provide these grids; it is possible to draw the frame and Merz grid (compose of two semicircles) on a conventional transparent paper and superpose them on the live or captured spermatozoon images.

Conclusion

High doses of metronidazole but not the therapeutic doses can decrease the length of all parts of the spermatozoon tail in mice. Spermatozoon count and motility and testosterone serum level are decreased by both high and therapeutic doses of metronidazole. Curcumin has an ameliorative effect on the spermatozoon count and motility, testosterone serum level and tail length in metronidazole-treated mice. Treatment of mice with curcumin can increase the spermatozoon motility.

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