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Original Article

Short and long term effects of different doses of paracetamol on sperm parameters and DNA integrity in mice

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ABSTRACT

The aim was to survey the impact of normal and high doses of paracetamol consumption on sperm parameters and DNA integrity in mice. A total of 36 adult male mice were divided into three groups: mice of group A served as control fed on basal diet, group B received normal dosage of Paracetamol (66 mg kg/day) and basal diet, group C received high dosage of Paracetamol (100 mg kg/day) and basal diet for 35, 70 and 105 days. The cauda epididymitis of each mouse was dissected and placed in 1 ml of pre-warm Ham's F10 culture medium for 20 min. The swim-out spermatozoa were analyzed for count, motility, morphology and viability. Sperm chromatin quality was evaluated by chromomycin A3 staining (CMA3), aniline blue staining and sperm chromatin dispersion test (SCD). The results showed that almost all of the sperm parameters significantly decrease following consumption of normal and high dosage of Paracetamol in three periods of experiments in mice ($p < 0.05$). Regarding to SCD test, we found a highly significant difference only in dose effect, but in CMA3 test and aniline blue staining there was a significant difference ($p < 0.05$) in both dose and time effects. According to our results, paracetamol as an analgesic and antipyretic may have detrimental effects on sperm parameters and DNA integrity in mice.

1. Introduction

Infertility is a major problem in up to 15% of the sexually active population and male factor is responsible in 50% of these cases [1]. As far as, lifestyle has some directly impresses on male infertility, sperm DNA quality and sperm parameters [2], we study paracetamol as a common pain killer. Acetaminophen (paracetamol) is widely used as an analgesic and antipyretic without prescription. The most commonly used over-the-counter (OTC) pain medication is acetaminophen along with aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) [3]. Paracetamol has a range of action like that of NSAIDs and be similar to particularly the COX-2 selective inhibitors. It is usually accepted that it inhibits COX-1 and COX-2 through metabolism by the peroxidase function of these isoenzymes. This results in inhibition of phenoxyl radical formation from a critical tyrosine remains needed for the cyclooxygenase activity of COX-1 and COX-2 and prostaglandin (PG) synthesis. Paracetamol is, on average, a weaker analgesic than NSAIDs or COX-2 selective inhibitors but is often chosen because of its better tolerance [4].

It is shown that overdosing with acetaminophen can cause hepatic necrosis in both humans and laboratory animals [5], and prolonged human use has been implicated in chronic renal disease [6] necrotic changes in lung [7], testis, lymphoid tissue of mice [8] and asthma in children [9]. Moreover, genotoxic effects of acetaminophen have been observed [10]. In vivo, acetaminophen has been shown to cause chromosome aberrations in bone marrow cells from exposed mice [3]. High doses of acetaminophen have also been reported to lead to testicular atrophy and decrease of testosterone hormone in vitro in rat and human [11–13].

There is a clear negative relationship between sperm chromatin/DNA damage and reproductive outcomes. Furthermore, it is generally accepted that the sperm chromatin condensation has a key role in male fertility, early embryonic growth and pregnancy outcomes [14]. The inter and intra molecular disulphide bonds of protamine molecules are essential for sperm nuclear compaction and stabilization. It is believed that this kind of nuclear compaction protects sperm genome from external damages including oxidative stress; temperature height and acid-induced DNA denaturation [15]. There are some kinds of tests for sperm chromatin/DNA evaluation which show different forms of damages. Chromatin struc-

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tural probes by nuclear dyes with cytochemical bases are sensitive, easy and economical which do not need exclusive device like flow cytometry [16]. There are some studies that showed effects of paracetamol on sperm parameters in mice and Rat [3,12,17]. Hence, we designed this study for the first time to evaluate Short and long term effects of different doses of paracetamol on sperm parameters and chromatin condensation in three period of spermatogenesis in mice by cytocemical tests.

2. Materials and methods

2.1. Animal

In this experimental study, totally 36 adult male NMRI mice with average weight 37 g and 11 ± 0 weeks old which were obtained from the animal house of Research & Clinical Center for Infertility of Shahid Sadoghi University of Medical Sciences. These mouse were divided into 3 groups; control (group I, n = 12), normal dosage (group II, n = 12) and high dosage (group III, n = 12) and each group was treated during three different periods with considering of mice spermatogenesis duration. Hence each group was divided into three subgroups (each n = 4) by 35, 70 and 105 days with or without treatments. Group II received normal dosage of acetaminophen which was calculated by the formula

$$D = MTR \quad (1\text{-}1)$$

where M is maximum recommended daily human dosage in tablets, R is ratio of weight of rat to average adult human weight of 60 kg and Tis weight of each tablet [18]. So, 66 mg/kg body weight acetaminophen was dissolved in daily water. The last group was given 100 mg/kg acetaminophen [19], but, in control group, we did not receive any medication. As we know acetaminophen is very bitter is the reason why we dissolved 0.002% and 0.003% saccharine in the daily water of group II and III. During experiments, animals were kept in standard condition with a temperature range of 25 ± 3 °C and mean relative humidity of 50 ± 5% in the animal house. This experimental study was approved by ethical committee of clinical center for infertility Shahid Sadoghi University.

2.2. Epididymal sperm preparation

We studied the spermatozoa after 1, 2 and 3 durations of spermatogenesis following drug treatments [21]. So, the mice were killed after 35, 70 and 105 days by cervical dislocated and the cauda epididymis of each animal was cut and placed in 1 ml Ham'sF10 medium. The dishes were incubated at 37 °C and 5% CO2 for 10 min to make spermatozoa swim out [21].

2.3. Sperm analysis

The sperm count, motility, normal morphology and viability (%) were evaluated for at least 200 spermatozoa from each animal. Sperm count and motility were evaluated by Meckler Chamber (Sefi Medical Co., Haifa) and light microscopy (Olympus Co., Tokyo, Japan). Motility indices were expressed as the percentages of progressive motility (rapid and slow), non-progressive and immotile spermatozoa. The morphologically normal spermatozoa and the percentage of viable sperm cells were assessed by Papanicolau staining and Eosin test respectively [22].

2.4. Sperm chromatin and DNA study

Sperm DNA integrity and chromatin condensation were assessed using Sperm Chromatin Dispersion (SCD) test and chromomycin A3 (CMA3) staining respectively.

2.4.1. Chromomycin A3 staining

CMA3 is a fluorochrome antibiotic which competes with the protamines for binding to the minor groove of DNA and show protamine deficiency. Briefly, the smears were dried and fixed in Carnoy's solution at 4 °C for 10 min. The slides were treated with 150 µl of CMA3 (0.25 mg/ml) in McIlvain buffer for 20 min. After staining in darkroom, they were washed in buffer and mounted with buffered glycerol. In each sample, at least 200 spermatozoa were counted under fluorescent microscope with a 460-nm filter and 100X eyepiece magnification and the percentage of $CMA3^+$ spermatozoa was reported. Bright yellow-stained chromomycin-reacted spermatozoa ($CMA3^+$) were considered as abnormal and yellowish green-stained or no reacted spermatozoa ($CMA3^-$) were considered as mature sperm with normal protamination [23].

2.4.2. Sperm chromatin dispersion (SCD) test

We used the sperm chromatin dispersion test for the assessment of sperm DNA fragmentation. The SCD test was performed via the Halosperm® Kit (INDAS laboratories, Madrid, Spain). Briefly, after adding 50 µl of semen to 100 µl of low melting agarose, 8 µl of this suspension was decanted on coated slide of the kit. A small lamella was put on it and kept on 4 °C for 5 min. The slide was immersed in Denaturant Agent (for 7 min) and Lysis Solution (for 20 min). After dehydrating, the slides were stained with Staining Solution A & B for 7 min [24].

2.4.3. Aniline blue

Aniline blue staining was indicated the surplus histone in chromatin structure. After preparing sperm and spread onto glass side, wait to air-dry. The smears slides were fixed in 3% buffered glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 30 min at room temperature. Afterwards, the smears slides were stained by aniline blue solution for 15 min.

3. Calculation

Statistical analysis was performed by SPSS version 20 for Windows (SPSS Inc., Chicago, IL, USA). Repeated measures analysis of variance (ANOVA) was applied to evaluate the significant differences between dose and time effects. The term 'statistically significant' was used to signify a two- sided P-value <0.05 for sperm parameters and cytochemical tests. All data were expressed in mean ± SD.

4. Results

It is significant to be mentioned that our study was performed in three different periods of spermatogenesis and the p-value of Mauchly's Test of Sphericity were more than 0.05 in all obtained results. Hence, we could use repeated measures of variance (ANOVA) with three levels (three different periods) as the most suitable test. As far as we were willing to figure out the effect of two different subjects effect (time and dose) on sperm quality, two-way repeated measures of variance analysis was performed. Summary of this analysis results was expressed separately in two different tables.

Table 4.1 demonstrates means, standard deviation and pairwise comparisons of sperm parameters. The part of pairwise comparisons in Table 4.1 has two subparts. One of them illustrates the comparison among three levels of time (one, two, and three periods of spermatogenesis). The results of this part will be able to answer a question that acetaminophen consumption for three periods of spermatogenesis can affect male fertility more than using it for two or less periods of spermatogenesis. A significant change between using paracetamol for one and three period of spermato-

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Table 4.1
Semen analyses data showing means, standard deviations & pairwise comparisons.

Parameters		Control (group A) Mean ± SD	66 mg kg/day normal dosage (group B) acetaminophen Mean ± SD	100 mg kg/day high dosage (group C) Acetaminophen Mean ± SD	Pairwise comparisons				
					Time (1, 2, 3)	p. Value		Treatment (group A, B, C)	p.Value
Count ($\times 10^6$)	1	11.25 ± 0.96	7.5 ± 1.29	6.0 ± 1.41	2	0.173		B	0.004*
	2	10.25 ± 0.96	8.5 ± 0.58	6.75 ± 1.26	1			A	
	3	10.75 ± 0.5	6.5 ± 2.4	5.0 ± 1.41	3	0.423		C	0.022*
Viability	1	70.75 ± 3.4	67.5 ± 2.08	65.25 ± 3.30	2	1.000		B	0.000*
	2	79.75 ± 4.11	61.75 ± 3.95	60.25 ± 4.5	1			A	
	3	70.25 ± 0.96	58.25 ± 3.40	55.75 ± 7.04	3	0.042*		C	0.007*
Rapid motility (%) (grade a)	1	20.25 ± 1.5	6.0 ± 1.82	4.75 ± 0.5	2	0.723		B	0.001*
	2	20.25 ± 1.25	8.75 ± 1.71	4.75 ± 2.5	1			A	
	3	34.0 ± 3.37	5.0 ± 0.82	3.5 ± 0.58	3	0.044*		C	0.000*
Slow motility (%) (grade b)	1	16.5 ± 2.38	15.25 ± 0.96	8.0 ± 1.15	2	0.216		B	0.003*
	2	16.0 ± 2.16	8.0 ± 1.41	4.25 ± 2.63	1			A	
	3	13.0 ± 2.45	5.0 ± 0.82	4.25 ± 1.71	3	0.032*		C	0.006*
Non progressive motility (%) (grade c)	1	28.5 ± 2.64	13.75 ± 2.06	26.5 ± 3.70	2	1.000		B	0.602
	2	25.5 ± 1.29	21.25 ± 2.5	22.5 ± 1.29	1			A	
	3	22.0 ± 3.56	36.5 ± 5.74	36.0 ± 2.71	3	0.010*		C	0.026
Immotile sperm (%) (grade d)	1	34.75 ± 2.75	64.25 ± 0.96	60.75 ± 4.79	2	0.134		B	0.001*
	2	38.25 ± 2.21	62.75 ± 1.71	68.5 ± 2.08	1			A	
	3	31.0 ± 2.16	53.5 ± 4.79	56.25 ± 3.86	3	0.025*		C	0.001*
Normal morphology	1	58.0 ± 2.16	44.75 ± 2.5	40.5 ± 2.08	2	1.000		B	0.003*
	2	58.0 ± 2.83	45.0 ± 2.94	36.25 ± 4.272	1			A	
	3	58.0 ± 3.46	33.25 ± 4.42	48.0 ± 3.83	3	1.000		C	0.004*
	2	30.25 ± 4.79	47.50 ± 7.85	52.75 ± 5.38					
	3	30.50 ± 5.45	52.25 ± 7.54	59.75 ± 5.74					

1 After one period of spermatogenesis.
2 After two period of spermatogenesis.
3 After three period of spermatogenesis.

Table 4.2
DNA and chromatin assays with means, standard deviations & pairwise comparisons.

Parameters		Control (group A) Mean ± SD	66 mg kg/day normal dosage (group B) acetaminophen Mean ± SD	100 mg kg/day high dosage (group C) Acetaminophen Mean ± SD	Pairwise comparisons				
					Time (1,2,3)	p. Value		Treatment (group A, B, C)	p.Value
SCD test	1	53.75 ± 2.99	71.5 ± 3.70	67.5 ± 6.24	2	0.636		B	0.012*
	2	52.25 ± 5.12	72 ± 5.94	77.75 ± 3.77	1			A	
	3	50.25 ± 2.06	66.25 ± 4.19	72.00 ± 5.29	3	1.000		C	0.001*
AB staining	1	41.25 ± 2.99	49.75 ± 5.38	57.5 ± 4.79	2	0.111		B	0.378
	2	42.75 ± 4.99	63.5 ± 4.65	70.75 ± 5.74	1			A	
	3	48.25 ± 0.5	57.25 ± 2.22	61.5 ± 2.64	3	0.023*		C	0.004*
CMA3 staining	1	24.25 ± 3.30	36.75 ± 6.13	48.75 ± 7.85	2	0.457		B	0.009*
	2	30.25 ± 4.79	47.50 ± 7.85	52.75 ± 5.38	1			A	
	3	30.50 ± 5.45	52.25 ± 7.54	59.75 ± 5.74	3	0.022*		C	0.012*

1 After one period of spermatogenesis.
2 After two period of spermatogenesis.
3 After three period of spermatogenesis.

genesis is observed in viability, and all parameters which related to motility of sperm. On the contrary, no significant differences are reported in count and normal morphology. The other part of pairwise comparisons gives up genuine comparison among three doses of treatments (group A, B, and C). It is clear that almost all of the sperm parameters except non-progressive blue staining had significant differences ($P < 0.05$) between three groups. The data showed that acetaminophen could impair sperm parameters after one or more spermatogenesis periods even in normal dosage.

The results of DNA tests by pairwise comparisons are displayed in Table 4.2. The comparisons of the results of aniline blue and CMA3 in three various periods of spermatogenesis show statistically significant changes. While, there is no meaningful different among SCD test results in all times. As we mentioned above, the second part of pairwise comparisons reveals critical information about three dosage of drug treatments. The eye-catching related point to be heeded is that approximately all of the DNA assays show significant differences between three groups.

Tables 4.3 and 4.4 indicates the summery of the tests within subject's effects among sperm parameters and DNA tests respectively. The effect of time, dose, and both elements were analyzed for each parameter of male fertility. The results of analysis indicate

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Table 4.3
Semen analyses with summery of the tests of within subjects effects.

Source		Sum of squares	Degree of freedom	Mean square	F ratio	p. Value	Partial eta squared
Count ($\times 10^6$)	Treatment	145.72	2	72.86	28.00	0.001*	0.903
	Error (treatment)	15.61	6	2.60			
	Time	7.72	2	3.86	4.68	0.059	0.610
	Error (time)	4.94	6	0.824			
	Treatment * Time	8.44	4	2.11	1.14	0.384	0.275
	Error (treatment * time)	22.22	12	1.85			
Viability	Treatment	1202.17	2	601.08	59.83	0.000*	0.952
	Error (treatment)	60.27	6	10.05			
	Time	302.17	2	151.08	13.41	0.006*	0.817
	Error (time)	67.61	6	11.27			
	Treatment * Time	281.66	4	70.42	4.95	0.014*	0.623
	Error (treatment * Time)	170.56	12	14.21			
Rapid motility (%) (grade a)	Treatment	3033.5	2	1516.75	355.33	0.000*	0.992
	Error (treatment)	25.61	6	4.27			
	Time	96.17	2	48.08	21.20	0.002*	0.876
	Error (time)	13.61	6	2.27			
	Treatment * Time	442.33	4	110.58	32.19	0.000*	0.915
	Error (treatment * Time)	41.22	12	3.43			
Slow motility (%) (grade b)	Treatment	567.39	2	283.69	81.70	0.000*	0.965
	Error (treatment)	20.83	6	3.47			
	Time	210.89	2	105.44	15.30	0.004*	0.836
	Error (time)	41.33	6	6.89			
	Treatment * Time	77.44	4	19.36	14.83	0.000*	0.832
	Error (treatment * Time)	15.67	12	1.31			
Non progressive motility (%) (grade c)	Treatment	126.00	2	63.00	21.00	0.002*	0.875
	Error (treatment)	18.00	4	3.00			
	Time	578.17	2	289.08	46.67	0.000*	0.940
	Error (time)	37.167	6	6.19			
	Treatment * Time	966.33	4	241.38	20.27	0.000*	0.871
	Error (treatment * Time)	143.00	12	11.91			
Immotile sperm (%) (grade d)	Treatment	5564.22	2	2782.11	397.44	0.000*	0.993
	Error (treatment)	42.0	6	7.00			
	Time	570.06	2	285.03	65.36	0.000*	0.956
	Error (time)	26.17	6	4.36			
	Treatment * Time	113.44	4	28.36	2.77	0.077	0.480
	Error (treatment * Time)	123.00	12	10.25			
Normal morphology	Treatment	2235.39	2	1117.69	90.96	0.000*	0.968
	Error (treatment)	73.72	6	12.29			
	Time	14.22	2	7.11	0.532	0.613	0.151
	Error (time)	80.22	6	13.370			
	Treatment * Time	629.44	4	157.36	15.05	0.000*	0.834
	Error (treatment * Time)	125.44	12	10.45			

Table 4.4
DNA analysis with summary of the tests of within subjects effects.

Source		Sum of squares	Degree of freedom	Mean square	F ratio	p. Value	Partial eta squared
SCD	Treatment	2950.89	2	1475.44	88.53	0.000*	0.967
	Error (treatment)	100.00	6	16.67			
	Time	127.05	2	63.53	3.67	0.091	0.550
	Error (time)	103.83	6	17.30			
	Treatment * Time	189.94	4	47.48	2.13	0.139	0.416
	Error (treatment * Time)	267.17	12	22.26			
AB staining	Treatment	819.39	2	409.69	18.88	0.003*	0.863
	Error (treatment)	130.17	6	21.69			
	Time	1177.56	2	588.78	18.79	0.003*	0.862
	Error (time)	188.00	6	31.33			
	Treatment * Time	1779.79	4	444.94	28.91	0.000*	0.906
	Error (treatment * Time)	184.67	12	15.39			
CMA3 staining	Treatment	4035.05	2	2017.52	60.31	0.000*	0.953
	Error (treatment)	200.72	6	33.45			
	Time	732.05	2	366.03	8.50	0.018*	0.739
	Error (time)	258.39	6	43.06			
	Treatment * Time	120.61	4	30.153	0.860	0.515	0.223
	Error (treatment * Time)	420.94	12	35.08			

that some parameters have a highly significant differences in all effects but the dose is more prominent (has larger partial eta number) than time and treatment * time such as viability, aniline blue staining, and mobility parameters. This table reveals that time does not affect on some others parameters like count and SCD test. Although, time does not influence in normal morphology, it can

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enhance the role of treatment. The data show that long-term use of acetaminophen in normal and high dosages causes damage to sperm quality and DNA integrity and increases sperm DNA fragmentation and protamine deficiency in mice.

5. Discussion

paracetamol as an analgesic and antipyretic without prescription commonly used during pregnancy and no significant relations were seen between acetaminophen use and low birth weight or preterm delivery and it was safe [25,26]. In some cases Paracetamol caused hematological effects [17], increased childhood wheeze and asthma risk [9], hepatotoxicity [20], multiple endocrine disturbances in the human adult testis [11].

The findings of present study showed that almost all of the sperm parameters were decreased following consumption of normal and high dosage of paracetamol in three period of spermatogenesis in mice. A similar study has shown that the treatment paracetamol causes a significant decrease in sperm motility and sperm count in rat that is in accordance with our results. It is also shown that paracetamol may cause a significant decrease in morphologically normal spermatozoa. These effects could be due to impacts of paracetamol on testis and epididymis [17]. Beside, Aksu et al. claim that their study demonstrates decrease in sperm motility and live sperm rate in male rat by consumption of paracetamol [27].

Ratnasooriya et al. declared that long-term administration of high doses of paracetamol damages the reproductive competence of male rats. They presented that this effects are reversible and was not due to a general toxicity but due to an increase in oligozoospermia, deficiencies of normal and hyper-activated sperm motility, and reduction in the fertilizing potential of spermatozoa [28]. In our study we also saw that long term consumption of paracetamol may disrupt sperm fertility potential.

To the best of our knowledge, this is the first investigation on the relationship between sperm chromatin condensation and short and long-term paracetamol administration in normal and high doses. Regarding to the DNA integrity tests, firstly in SCD test, we found a significant difference among groups in three period of spermatogenesis. This showed that paracetamol consumption in normal and high doses may cause sperm DNA fragmentation. Secondly, In CMA3 staining, in the first 35 days we found significant differences between groups only when high dosage of paracetamol was treated. On the other hand, there were significantly differences between groups in the rates of sperm protamine deficiency in both normal and high dosage of paracetamol after 70 and 105 days. Thus, it seems that long-term use of paracetamol has detrimental effects on histone–protamines replacement during the testicular phase of sperm chromatin packaging and cause sperm protamine deficiency in mice. To compare our data with others, although we did not find any similar study in the literature, but, there was a study on testicular cells populations with tetraploid, diploid, and haploid DNA content using acridine orange staining with flow cytometry in mice. The results of mentioned study showed that daily treatments with either hydroxyurea or acetaminophen caused an increased frequency of cells with altered sperm chromatin structure and increased sperm DNA denaturation in mice [3] which confirmed our results.

There are some possible reasons for sperm DNA damage following acetaminophen treatments. It is shown that acetaminophen causes an inhibition of both DNA replication and DNA repair by a specific inhibition of enzyme ribonucleotide reductase [10]. In fact, these effects provide a reason for acetaminophen's ability to make sister chromatid exchanges, micronuclei, chromosomal aberrations and apoptosis in cells with low drug metabolism activity [3]. On the other hand, hepatotoxic dose of acetaminophen in mice was accompanied by a dramatic decrease in mRNA for histone [3,6]. So, it is possible that the altered chromatin structure observed in the sperm could be related to errors in histone synthesis and fallowed by disruption in histone–protamines replacement during the testicular phase of sperm chromatin packaging. Further studies are required to illuminate the mechanism of action and the effects of different dosage of acetaminophen on human spermatozoa.

6. Conclusion

Our study showed that paracetamol or acetaminophen as an analgesic and antipyretic drug may impact sperm parameters and chromatin/DNA integrity in mice. It should be noted that these effects are dose dependent and are seen both in short and long-terms of drug consumption. We advise that men especially in fertility period be careful in acetaminophen usage without prescription of their physician.

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