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Spermatotoxic Effect of Methanol Extract of *Quassia amara* L: Impact on Expression of Specific Genes Concerned with Ubiquitination-Proteosome Degradation Pathway

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Abstract

Ubiquitination is believed to play a critical role in removal of dead and/or defective spermatozoa in normal and, more importantly, under circumstances when such spermatozoa are produced in large numbers due to genetic defects or toxic manifestations. Ubiquitination under such instances would involve specific gene expressions, many of which are not yet clearly understood. In an exhaustive study in Swiss mouse model to find the spermatotoxic effect of quassin, a diterpene compound isolated from *Quassia amara*, we found most of the spermatozoa to be abnormal in morphology and unviable. In the present study, we aimed at analysing the transcriptional profile of three selected genes, *Ubb, Ube2c* and *Psmb8*, involved in the ubiquitin proteolytic pathway in the testis and epididymal segments of *Q. amara* bark methanol extract treated mice adopting semi-quantitative RT-PCR and to study the level of DNA damage of the treated mouse spermatozoa. The results revealed that the treatment induced considerable damage to the sperm DNA. All the three genes studied showed marked increase in their levels of expression in the treated mice compared to the corresponding controls. Thus, this study shows that *Q. amara* methanol extract is causative of sperm DNA damage and defective spermatozoa and, in such cases, the expression of specific genes concerned with ubiquitination pathway is increased, implying that ubiquitination-proteosomal degradation is involved in the processing of dead/defective spermatozoa.

Keywords: Defective Sperm, Proteosomal Degradation, Quassin, *Quassia amara*, Spermatotoxicity, Ubiquitination

1. Introduction

Mammalian spermatozoa undergo morphological, biochemical, and physiological modifications initially in the testis (testicular maturation) and later in the epididymis (epididymal maturation). These maturational processes require regulated proteolysis and organelle degradation that are mediated by several proteins which undergo post-translational modifications such as phosphorylation, glycosylation, acetylation, etc. One of the most important post-translational modifications of proteins is ubiquitination, which involves attachment of one or more ubiquitin monomers to the protein moiety through covalent bond. Several experimental findings support a link between the ubiquitin-dependent pathway of protein degradation and spermatogenesis[7,15]. The complex system of ubiquitination is involved in the control of a multitude of processes, including cell cycle progression, DNA repair, protein degradation and recycling, protein synthesis and processing, regulation of transcription, membrane trafficking, cell signalling, apoptosis, stress responses, and diseases[34].

In the male reproductive system, the ubiquitin-proteosome pathway fulfils necessary requirements for the substrate specificity and developmental programming of proteolysis within the differentiating male germ cells[18,53,49,24]. Certain ubiquitin genes such as ubiquitin, ubiquitin conjugating enzymes E1, E2 and UBC4 and the ubiquitin-recycling protein were found to be highly expressed during gonad and germ cell differentiation

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by Sertoli cells, spermatogonia, spermatocytes and spermatids[52,30,54,55] have localized the ubiquitin in mitochondria of spermatids and mature sperm. Expression of ubiquitin in the round spermatids and mature sperm has been reported in roosters[1], bulls[55] men and mice[62]. The ubiquitin-activating enzyme E1[23] and ubiquitin carriers E2[64,55] and UBC4[36,65] are active during spermatogenesis. Also, ubiquitination plays a role in the replacement of spermatid's nuclear histones by transition proteins, followed by permanent substitution with protamines during spermatid elongation. Histone H2A[4] and histone H3[10] are ubiquitinated and discarded in the cytoplasmic droplet prior to sperm release from seminiferous tubules. Proteasomal subunits were also detected inside or near sperm centriole[8,32,66]. Ubiquitin is present in human seminal plasma[28] and the defective spermatozoa in humans as well as animals become ubiquitinated during epididymal passage[56,57,49,24].

A portion of the defective spermatozoa which are ubiquitinated are removed as they descend down the corpus and cauda epididymides by phagocytosis, and the other portion of the defective spermatozoa which escape phagocytosis are present in the ejaculate[56,24]. Further[56], noticed ubiquitination of the cytoplasmic droplets on the sperm mid-piece and reabsorbtion of the same during epididymal passage. Precisely, the percentage of ubiquitinated sperm increases during their transit from rete-testis to corpus of epididymidis, and decreases from corpus to cauda of epididymidis. Observed ubiquitination of sperm mitochondrial membrane proteins, such as prohibitin, during spermiogenesis which are masked by disulfide bond cross linking during epididymal passage[54,12]. Although it is likely that defective sperm are ubiquitinated because of structural damage, the mechanism by which such sperm are recognized by the ubiquitination machinery, and the means of disposal after phagocytosis, are not yet clearly understood. One possible explanation could be that the process of ubiquitination of epididymis is at the intersection, or perhaps at the common end-point of several apoptotic mechanisms operating in the testis[48] and such mechanisms may recognize structural damage of sperm[42] and/or damaged sperm accessory structures. Suggested that a whole sperm cell can become ubiquitinated on its surface and eventually phagocytosed[56]. Similar to the proteolysis of endocytosed receptors[51] destruction of the phagocytosed sperm seems to occur by means of lysosomal/vacuolar proteolysis.

Mammalian genomes have four functional Ub genes: two Ub-ribosomal fusion genes viz., *Uba52* (accession no. MGI:98887) and *Uba80* (accession no. MGI:1925544, also known as *Rps27a*), and two polyubiquitin genes, *Ubb* (accession no. MGI:98888) and *Ubc* (accession no. MGI:98889), consisting of four and nine tandem Ub coding units, respectively[29,63,5,6,16,38]. The relative contribution of each of these Ub genes to basal Ub levels varies widely among different tissues and cell types[40]. The mouse polyubiquitin gene UbC is essential for fetal liver development, cell-cycle progression and stress tolerance[40]. Another polyubiquitin gene, *Ubb,* plays a critical role in germ cell development and meiosis[41]. *Ube2b* gene codes for ubiquitin-conjugating enzyme E2. *Ube2b*$^{-/-}$ mice were found to exhibit male infertility, sperm head anomalies and characteristic abnormal distribution of periaxonemal structures[25]. In mammals the highest rate of ubiquitination is found in testis, and it increases in testes containing haploid spermatids[36,37]. The 26S proteasome activity during mouse spermiogenesis reaches the peak at the stage of elongating spermatids when ubiquitin–protein conjugates are the highest[62]. Components of the 26S proteasome are present in the manchette and also can be isolated from rat sperm tail[32,24].

The mechanism underlying ubiquitination of abnormal spermatozoa is not yet clearly understood. One possibility is that the apoptotic, or otherwise compromised, spermatozoa with altered plasma membranes; permeabilized mitochondrial membranes and fragmented nuclear DNA are recognized by ubiquitin. While only 20-40% of defective, ubiquitinated spermatozoa carry fragmented DNA, the results of Sutovsky et al.[58] demonstrate a high degree of correlation between sperm ubiquitination and DNA defects. While almost all spermatozoa with defective nuclear DNA are subject to ubiquitination, many other ubiquitinated spermatozoa with visible defects do not carry fragmented DNA. These findings suggest that the spermatozoa with chromatin/DNA defects are recognized in the epididymis and marked for destruction by ubiquitin-dependent proteolytic pathway[58,31,45].

Since in an earlier study we found treatment of male Swiss mouse with quassin, a seco-triterpenoid compound from *Quassia amara* L. bark, produced severe male reproductive toxic effect wherein the incidence of abnormally-shaped spermatozoa was very high[14], it was found pertinent to find if the ubiquitin-proteolytic pathway is augmented under such circumstances. Our

approach here was to find the expression profile of three selected genes, Ubb, Ube2c and PSMB8, concerned with ubiquitination, in the testis and different segments of the epididymis. Thus, in the present study, we analyzed the transcriptional profile of the three selected genes involved in the ubiquitin-proteolytic pathway in the testis and epididymal segments of *Q. amara* methanol extract treated mice. The mice were also studied for the level of DNA damage, since these spermatozoa would also be removed through ubiquitination – proteosomal degradation.

2. Materials and Methods

2.1 *Quassia amara* Extract

The procudre of extraction of *Q. amara* bark in methanol has been explained in our earlier paper[14]. The bark of *Q. amara* was received as a generous gift from Late Dr. BMJ Pereira, IIT Roorkee, Uttaranchal, India. Approximately 200g of the powdered bark of the plant was extracted for about 20 cycles, in 1L of methanol using a Soxhlet apparatus. The extract was dried in a rotary evaporator and further condensed by air drying at room temperature, to yield approximately 2g of crude extract. This was stored at 0 ^{0}C till use. The condensed methanol extract was dissolved in minimum quantity of ethanol and further diluted with phosphate buffered saline (PBS).

2.2 Experimental

Twenty 90 day old male Swiss albino mice, each weighing 30-35 g, raised from a stock obtained from Indian Institute of Science, Bangalore, India, were divided into two groups of 10 each. A control group was administered with PBS, and the second group was injected with methanol extract at a daily dose of 100 mg/kg body weight, through intraperitoneal (*ip*) route for 35 days, the duration of one spermatogenic cycle in mouse[35]. At the end of the treatment, mice in each group were dissected under mild sodium pentathol anaesthesia and the testicles and epididymides were removed. Tissues form five animals from each group were used for RNA extraction and five animals were used for COMET analysis.

2.3 RNA Isolation

RNA was isolated from 20-30 mg of testicular and caput, corpus and cauda epididymidal tissues of *Q. amara* extract-treated and control mice by acid guanidinium thiocyanate-phenol-chloroform method[11]. The RNA samples were quantified in a Perkin-Elmer spectrophotometer (USA) at 260 nm. All the isolated RNA samples had an A260/A280 ratio of 1.8-2.2. RNA samples were analysed by separation in 1 % agarose gel[27] and stained with 0.5 µg/ml ethidium bromide. The RNAs were immediately stored as aliquots at -80 ^{0}C immediately after isolation.

2.4 Semi-quantitative Reverse Transcriptase – Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed using Qiagen (USA) one step RT-PCR kit as per the manufacturer's instructions. The primer sequences of the concerned genes are given in the table 1. *Actβ* (β-actin) was used as the endogenous control. The appropriate number of PCR cycles was performed so that the amplicon of both the target and reference genes are clearly visible on agarose gel and could be identified. Varied PCR conditions and primer concentrations were used for different genes according the melting temperature and other parameters of the respective primers used. The PCR products were resolved on a 2% agarose gel stained with ethidium bromide. The molecular size of the amplified products (CRP and GAPDH) was determined by comparison with molecular weight markers (100 bp DNA ladder, Genei, Bangalore, India) run in parallel with the RT-PCR products. Gels were subjected to densitometric scanning, and the band intensities of the cDNA fragments of the genes were normalized against the band intensity of cDNA fragment of the house-keeping gene *Actβ*, using Quantity One Software (Bio-Rad, CA, USA). The experiments were performed in tetraplicate. Student's *t* test was performed with CoStat software.

2.5 Comet Assay

Spermatozoa from the cauda epididymidis were collected in a clean petri dish containing PBS (pH 7.4). The slides

Table 1. PCR primers for semi-quantitative RT-PCR

Gene	Forward primer (5'-3')	T_m	Reverse primer (5'-3')	T_m	Accession# in Genebank
Psmb8	CTCTAGCTGGTTTCACTGATGTGGC	64.4	CCTCAGGAATGCGGTGGGC	65.4	NM_010724.2
Ubb	CCAGAAAGAGTCAACCCTGC	56.72	CACCTCCAAGGTGATGGTCT	57.02	NM_011664.3
Ube2c	AGGAGAACCCAACATCGATAGCC	63.55	GCTGTAGCCGCTTGCCCAC	63.85	NM_026785.2
Actb	CTGGGTCATCTTTTCACGGT	57.6	TGTTACCAACTGGGACGACA	56.69	NM_007393.3

were coated first with 1% normal melting point agarose in Ca^{2+} and Mg^{2+} free PBS. The second layer was prepared by layering 0.8% low melting agarose (Sigma Aldrich Chemicals Co., MO, USA) mixed with approximately 1 x 10^5 spermatozoa. The third layer of agarose of same concentration was added to fill any residual holes in the second layer and to increase the distance between cells and gel surface. The sperm membrane lysis and nuclear de-condensation were carried out in lysis solution containing 2.5 mM NaCl, 100 mM disodium EDTA, 10 mM Trizma base, and 1% Triton X-100, for overnight at 4 °C. Again, the slides were transferred to fresh lysing solution containing 15 mM dithiothreitol without Triton X-100 and kept at 4 °C for 2 hours. Alkaline unwinding was carried out in electrophoresis buffer for 30 minutes. Electrophoresis was carried out in buffer containing 10 N NaOH and 200 mM EDTA for 20 minutes under alkaline pH at a fixed voltage[46]. The cells were stained with 2 µg/ml ethidium bromide and observed in a fluorescent microscope (Carl Zeiss, Jena, Germany). The extent of damage in the sperm DNA was estimated by measuring the amount of tail DNA using CASP software[26].

3. Results

Expression profiles of the genes- The compounded relative gene expression wherein each gene was compared against *Actβ*:

Psmb8 transcript was not detected in the testis of control mouse but was found in the caput, corpus and cauda epididymides, in abundance in that order. In the *Q. amara* treated mice the transcript of this gene was prolific in the testis and the expression was heightened in the three segments of epididymis, but maintaining the region-specificity of abundance as in the control (Figure 1).

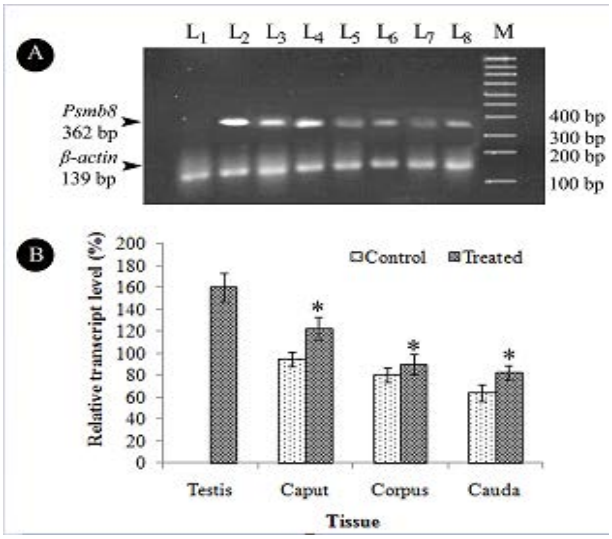

Figure 1. (**a**) Semi-quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) analysis of *Psmb8* mRNA transcript levels visualized on an ethidium bromide-stained agarose gel. M, 100bp DNA ladder; L1, control testis; L2, treated testis; L3, control caput; L4, treated caput; L5, control corpus; L6, treated corpus; L7, control cauda; L8, treated cauda. **(b)** The results depicted are normalized to levels of Actβ gene. Data are expressed as mean±SD. Value of ratios of intensity for *Psmb8* gene divided by that for Actβ (tested in tetraplicate). *Significance at $P < 0.05$

The expression of *Ubb* in the control mouse was in the order, testis > caput > corpus > cauda. In the treated mouse the expression was heightened in testis and little altered in the segments of epididymis (Figure 2).

On the other hand, *Ube2c* expression was limited to testis; there was no expression in the epididymis. Within this limitation, its expression was increased in the treated mouse (Figure 3).

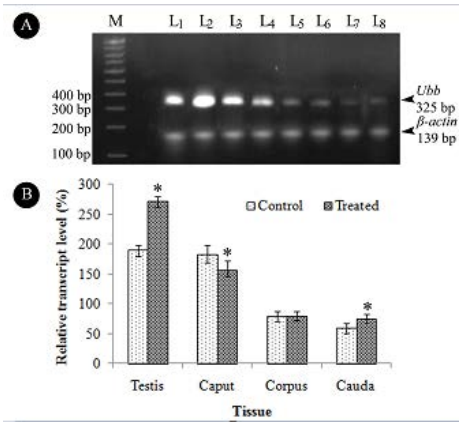

Figure 2. (**a**) Semi-quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) analysis of *Ubb* mRNA transcript levels visualized on an ethidium bromide-stained agarose gel. M, 100bp DNA ladder; L1, control testis; L2, treated testis; L3, control caput; L4, treated caput; L5, control corpus; L6, treated corpus; L7, control cauda; L8, treated cauda. (**b**) The results depicted are normalized to levels of Actβ gene. Data are expressed as mean±SD. Value of ratios of intensity for *Ubb* gene divided by that for Actβ (tested in tetraplicate). *Significance at $P < 0.05$

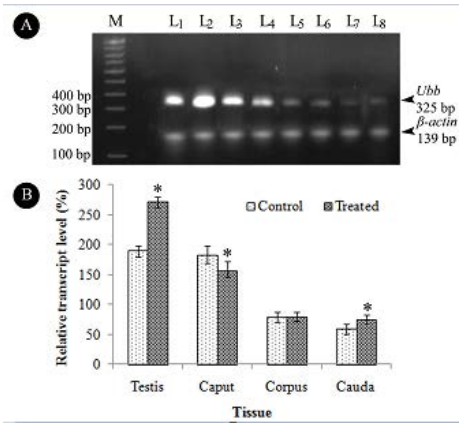

Figure 3. (**a**) Semi-quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) analysis of *Ube2C* mRNA transcript levels visualized on an ethidium bromide-stained agarose gel. M, 100bp DNA ladder; L1, control testis; L2, treated testis; L3, control caput; L4, treated caput; L5, control corpus; L6, treated corpus; L7, control cauda; L8, treated cauda. (**b**) The results depicted are normalized to levels of Actβ gene. Data are expressed as mean±SD. Value of ratios of intensity for *Ube2C* gene divided by that for Actβ (tested in tetraplicate). *Significance at $P < 0.05$

3.1 DNA Damage

In the comet assay, as is known, a cell with fragmented DNA had the appearance of a 'comet' with a bright fluorescent head and a fluorescent tail whose intensity represents the relative amount of DNA strand breaks[20,46]. Cells are generally assigned to five groups, 0-20% (intact), 20-40% (slightly damaged), 40-60% (damaged), 60-80% (highly damaged) and >80% (dead) (Maire et al., 2007). The results of the present study are presented in the tables 2, 3 and figures 4, 5, from which it is clear that the treatment induced slight damage and in rare cases high damage to the sperm DNA.

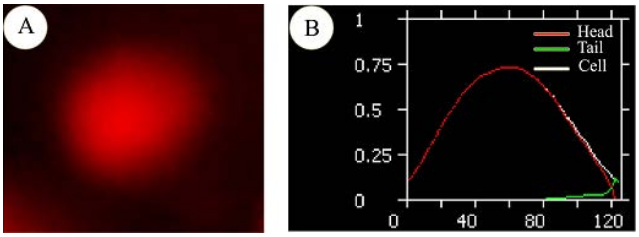

Figure 4. (**a**) Fluorescent photomicrograph of control mouse spermatozoon processed using Comet assay, shows intact DNA. (**b**) Profile of the comet as revealed in the CASP analysis.

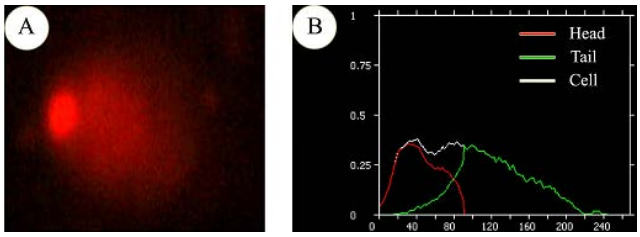

Figure 5. (**a**) Fluorescent photomicrograph of *Q. amara* treated mouse spermatozoon processed using Comet assay, shows fragmented DNA. (**b**) Profile of the comet as revealed in the CASP analysis.

4. Discussion

The present study demonstrates reprotoxic and spermatotoxic effect of *Q. amara* as revealed in the transcriptional profiling of selected gene products within the proteolytic ubqiuitin-proteosome pathway. From our histological study it is quite evident that *Q. amara* (*visa vi* quassin) induces several manifestations in the sperm morphology, viability and motility. Various abnormal morphologies such as head alone, tail alone, macrocephali, kinked head, retention of cytoplasmaic droplet, lasso-like sperm, coiling of tail, double-tail, etc., have also been identified[14]. As it has been previously established that sperm abnormalities and sperm surface ubiquitination are

interlinked[56], it is quite relevant to study the expression of genes involved in the UPP pathway. *Ubb*, one of the four ubiquitously expressed, seemingly redundant, genes, leads to an arrest of meiotic progression in male and female germ cells, as well as degenerative changes to the testes. Have generated mice harbouring a targeted disruption of the polyubiquitin B (*Ubb*) gene in order to assess the requirement for maintenance of adequate Ub supplies in cellular function and development[41]. The outcome of this study shows that mice lacking one or both copies of the *Ubb* develop normally and are viable at birth, but $Ubb^{-/-}$ mice of both genders are infertile. In case of $Ubb^{-/-}$ mice, the germ cells progressed to the pachytene stage and were found to form apparently normal synaptonemal complexes in both males and females. The $Ubb^{-/-}$ spermatocytes formed XY bodies containing ubiquitinated histones but ultimately underwent cell death during pachytene, and the mice exhibited complete testicular degeneration by two years of age. Thus, these findings suggest a critical role for *Ubb* in germ cell development and meiosis[41]. In the present study, it is found that the level of expression of *Ubb* is relatively high in the testis and little altered in the segments of epididymidis. It implies that the tendency of the testis towards meiotic arrest may be induced due to the treatment.

Have substantiated the presence of Ube2 in testis and epididymis[61]. In the testis, Ube2 was found to be localized in the residual body and cytoplasmic lobe, but also within the developing acrosome of round and elongating spermatids and in the chromatin of pachytene and dividing spermatocytes. In epididymis Ube2 was found to be localized as a distinct peri-nuclear focus in the principal cells, but the authors did not give any detail about the sub-class of Ube2, whether it is *Ube2c* or any other sub-class. But in our present study, *Ube2c*, which is expressed specifically in the testis, shows significant increase in the expression level when compared to the control. *Ube2c* catalyzes the covalent attachment of ubiquitin to other proteins and also is required for the destruction of mitotic cyclins[33]. Therefore, this elevated level of *Ube2c* in the testis reflects the increased destruction of mitotic cyclins which might be a preparation for the removal of defective spermatozoa or germ cells by the process of ubiquitination[25].

Table 2. Showing the details of comet assay of control mouse spermtozoa, as scored by CASP software

No. of	Length			Percentage	
cells	Head	Tail	Comet	Head DNA	Tail DNA
1	99	3	102	99.46	0.54
2	99	3	102	99.59	0.41
3	81	3	84	99.40	0.60
4	81	3	84	99.37	0.63
5	75	3	78	99.27	0.77
6	85	3	88	99.38	0.62
7	69	3	72	98.78	1.22
8	67	3	70	98.57	1.43
9	67	3	70	99.07	0.93
10	67	3	70	98.52	1.48
11	87	3	90	99.26	0.74
12	97	3	100	99.45	0.55
13	99	3	102	99.50	0.49
14	97	3	100	99.52	0.48
15	79	3	82	99.28	0.72
16	81	3	84	99.21	0.79
17	93	3	96	99.33	0.67
18	97	3	100	99.52	0.48
19	97	3	100	99.48	0.52
20	105	3	108	99.59	0.41
21	77	3	80	99.0	1.00
22	79	3	82	99.23	0.76
23	83	3	86	99.22	0.78
24	83	3	86	99.32	0.68
25	79	3	82	99.27	0.73
26	81	3	84	99.34	0.66
27	85	3	88	99.33	0.66
28	47	4	51	98.55	1.45
29	127	3	130	99.72	0.28
30	131	3	134	99.67	0.33
31	123	3	126	99.67	0.31
32	109	3	112	99.64	0.36
33	117	3	120	99.57	0.43
34	125	3	128	99.63	0.37
35	115	3	118	99.60	0.40
36	105	3	108	99.53	0.47
37	95	3	98	99.42	0.58
38	77	3	80	99.20	0.80
39	83	3	86	99.30	0.70
40	99	3	102	99.46	0.54
41	83	3	86	99.21	0.79
42	57	3	60	98.16	1.84
43	111	3	114	99.56	0.44
44	79	3	82	98.91	1.08
45	67	3	70	99.10	0.89
46	73	3	76	98.98	1.02
47	69	3	72	99.11	0.87
48	79	3	82	99.23	0.77
49	97	3	100	99.30	0.71
50	97	3	100	99.30	0.70

Table 3. Showing the details of comet assay of *Q. amara* treated mouse spermtozoa, as scored by CASP software

No. of cells	Length			Percentage	
	Head	Tail	Comet	Head DNA	Tail DNA
1	63	33	96	72.24	27.76
2	69	33	100	83.95	16.05
3	77	36	113	84.29	15.71
4	65	37	102	69.1	30.9
5	85	42	127	82.28	16.72
6	69	71	140	64.71	35.29
7	71	73	144	63.05	36.95
8	9	7	16	68.40	31.62
9	27	15	42	80.09	19.91
10	27	10	37	89.40	10.60
11	45	11	56	90.84	9.16
12	69	29	98	61.59	38.41
13	123	95	218	79.09	20.91
14	123	100	223	77.20	22.80
15	125	120	245	74.68	25.32
16	131	124	255	74.03	25.97
17	133	150	283	73.0	27.0
18	101	66	167	79.81	20.19
19	97	68	165	79.12	20.88
20	93	60	153	78.90	21.10
21	103	141	244	67.73	32.27
22	75	45	120	77.30	22.70
23	73	66	139	72.37	27.63
24	71	45	116	75.51	24.49
25	73	24	97	73.14	26.86
26	45	92	137	53.02	46.98
27	67	20	87	74.35	25.65
28	45	92	137	52.88	47.12
29	57	79	136	61.47	38.53
30	57	82	139	60.47	39.53
31	57	78	135	63.60	36.40
32	71	50	121	77.39	22.60
33	65	49	114	75.09	24.91
34	65	62	127	69.31	30.69
35	83	88	171	58.81	41.18
36	89	79	168	63.85	36.15
37	101	63	164	73.47	26.53
38	57	14	71	76.59	23.41
39	101	52	153	75.68	24.32
40	67	60	127	73.46	26.54
41	53	45	98	65.44	34.56
42	49	38	87	69.41	30.59
43	49	36	85	72.35	27.65
44	129	107	236	45.49	54.51
45	102	61	163	75.51	24.49
46	85	85	170	61.00	38.99
47	101	74	175	73.45	26.55
48	49	86	135	54.22	45.78
49	63	73	136	58.94	41.06
50	121	58	179	75.83	24.17

Psmb8, *Psmb9* and *Psmb10*, the inducible proteosomal core subunits replace their constitutive counterparts *Psmb5*, *Psmb1* and *Psmb2* in the professional antigen-presenting cells[39], but eye lens cells[47] and sperm acrosome are also known to have these core subunits. In the present study, the expression of *Psmb8* in the testis is found to be significantly increased than in the controls, and in the case of the epididymal segments the relative expression of this gene was higher in the caput and less in the corpus and further less in the cauda. But, found that the expression level of *Psmb8* is reduced in the testis and the corpus in theophylline-treated rats than in the controls[61]. Also, its expression level is elevated in the cauda of treated animals when compared to the controls, which is quite contrary to the observation in the present study. In addition to *Psmb8*, studied the expression pattern of the other two inducible subunits, *Psmb9* and *Psmb10*, levels of expression of which have been reported to be insignificant[61]. For this irrelevant pattern of expression among the proteosomal core subunits, the authors suggest that the loss of inducible 20S subunit transcripts could also be masked by an increased infiltration of white blood cells expressing inducible subunits in the testicular and epididymal tissues of exposed animals but, then, such a white blood cell infiltration has not been detected by their histological analysis. The expression of *Psmb1* after theophylline exposure was found to follow the order, testis > corpus > caput > cauda. And, also, the authors inferred that *Psmb1, Psmb2* and *Psmb5* and *Ube2d3* genes were affected significantly in the testis and corpus epididymidis, but the magnitude of effect differed slightly based on the administered toxicant. Such an altered gene expression may correlate with aberrant spermatogenesis in the testis, and impair the processing of both normal and defective spermatozoa in the epididymis. Thus, from the study, it could be concluded that most of the genes in the UPP pathway follow the expression pattern, testis > caput > corpus > cauda or caput > testis > corpus >cauda[61]. However, in the results of our study, transcriptional profile of the proteosomal core subunit, *Psmb8* and polyubiquitin gene, *Ubb* showed expression apparently in the following order: testis > caput > corpus > cauda as in the case of expression of *Ube1*, *Ube2d3* and *Uchl1*[61]. The gene encoding ubiquitin conjugating enzyme, *Ube2c* showed expression only in the testis. Thus, the pattern of transcriptional profile of the genes involved in UPP pathway observed in the present study is in

accordance with the established fact that the percentage of ubiquitinated sperm increases during the transit from rete-testis to corpus epididymidis, and decreases from the corpus to the cauda[56,17] and, hence, the higher occurrence of ubiquitination in the caput might be the reason for the increased expression of the studied genes in the caput compared to the other epididymal segments. Since proteosomes have also been detected in the epididymal fluid[22], it is possible that defective spermatozoa are partially degraded intra-luminally in the caput and corpus epididymides and the resident clear cells of the corpus epididymidis take up and degrade ubiquitinated proteins released from moribund spermatozoa. Such a mechanism of defective sperm ubiqutination in the caput and removal in the corpus epididymis is consistent with the transcriptional profiles of the selected genes in the individual epididymal segments in the present study.

This perception is further supported in a study using monoclonal antibody KM691[60]. These authors detected a basal level of ubiquitin cross-reactivity in the testis and epididymis of control rats. Though there was accumulation of ubiquitin in the cytoplasmic droplets present in the epididymal spermatozoa, the strong accumulation of ubiquitin pertained to the secretory sites such as stereocilia and apical blebs of the epididymal epithelial lining only in the treated rats. Using monoclonal antibodies against polyubiquitin chains MC034, these authors detected the presence of such chains in the testicular stroma and seminiferous tubules of both control and treated rats.

First observed that increased accumulation of ubiquitin on the surface of the defective mammalian spermatozoa correlates with poor sperm quality and infertility[56,55]. Further, these authors demonstrated a correlation between ubiquitination and DNA damage[45]. Performed TUNEL assay for the detection of single-stranded, apoptotic nuclear DNA on tissue sections of rat treated with theophylline, in combination with ubiquitin labelling by antibody KM691[60]. Neither control nor treated rats showed a substantial increase in apoptosis within the seminiferous tubules. The authors produced results indicating a qualitative increase in apoptotic cells in the testicular stroma of treated rats. Thus, it was suggested that apoptosis of the stromal Leydig cells could, in part, be responsible for changes in spermatogenesis seen in treated rats by conventional and histological analysis. Also, these authors noticed a slight increase in ubiquitin expression within the seminiferous tubules. Similar to the testis, a few apoptotic nuclei have also been detected in the epididymal tissue of control and treated rats. Interestingly, the cytoplasm of the phagocytic clear cells in the treated rats displayed a distinct TUNEL signal, probably due to endocytosis of the disintegrated cell nuclei and DNA from the epididymal tubule lumen.

Quite a few studies have adopted COMET assay to assess the DNA damage of the spermatozoa induced by several mutagenic agents[21] and toxicants[2,19,44,3] and also to evaluate the efficiency of various methods of cryopreservation in maintaining the integrity of the DNA[50,13,9,43]. We performed alkaline comet assay to study if *Q. amara* treatment induces DNA damage to the spermatozoa, if any, and to link ubiquitination with the DNA damage. The result of the comet assay reveals that there is considerable DNA damage in the treated spermatozoa. Therefore, the results reveal a positive correlation between DNA damage and ubiqutination.

Thus, this study establishes that i) *Q. amara* methanol extract (*visa vi* quassin) is spermatotoxic, causing DNA damage and also gene/physiological alteration during spermatogenesis and/or spermiogenesis, leading to altered sperm morphologies, and ii) the affected spermatozoa are processed testicularly and/or extra-testicularly with ubiquitin-proteosomal degradation as revealed in the profiling of the specific genes.

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