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Implication of Hongres1 Protein in Quassin-Induced Male Reproductive Abnormality in Rats

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Abstract

Objective: Quassin, the bioactive constituent of Quassia amara plant, has been reported to decrease sperm count, sperm motility, normal sperm morphology and male fertility index. These sperm characteristics are however acquired in the epididyimis. Information on the effects of quassin on epididymal functions is scanty. The effects of quassin on epididymal functions were therefore investigated in Wistar albino rats.

Methods: Quassin (0.1 and 2 mg/kg) and distilled water (0.5 ml) were administered by gavage (p. o) daily for 6 weeks to male rats (180-200 g, n=5), and thereafter sacrificed. Sperm motility, viability and count were examined microscopically. Serum from each rat was analyzed for Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH) and Testosterone by enzyme immunoassay technique. Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST) and Alkaline Phosphatase (ALP) were measured spectrophotometrically. Epididymal HongrES1 protein expression was assessed using immune histochemical technique. Data were analyzed using Student's t-test at p=0.05.

Results: Quassin significantly decreased sperm motility, % viability and count. Serum FSH, LH and testosterone were also decreased. The epididymal tubules were coiled and hyperplastic, while no visible lesion was observed on the testes. Serum ALT, AST, LDL, HDL, cholesterol, triglyceride, bilirubin and total protein levels were not affected by quassin. Total epididymal protein was however significantly reduced while total testicular protein increased. Epididymal HongrES1 protein expression was suppressed by treatment with quassin.

Conclusion: Quassin decreased epididymal tissue total protein and HongrES1 protein expression in rats. These effects indicate that antifertility action of quassin is pronounced in the epididymis.

Keywords: Quassin; Epididymal protein; Testes; HongrES1; FSH; LH; Testosterone

Introduction

Quassin is the major bioactive principle of Quassia amara plant which has been reported to be responsible for the reproductive toxicity of the plant [1]. Q. Amarais a 6-8 meters tall tree native to Suriname, Brazil, in South America. It is a member of the Simaroubaceae family of plants and its synonyms include suriname tree, amargo and bitter wood. Traditionally, the stem bark and leaves have been used in herbal remedies. Bioactive properties attributed with this plant in literature include as a bitter stomachic, antifungal, antiulcerative, anticarcinogenic, antiviral and amoebicidal [2,3]. Its use in the context of its potential biopesticidal application and antimalarial property were also reported [4,5]. Preliminary reports implicating this plant in male reproductive toxicology were first reported by Njar et al. [6], Raji and Bolarinwa [1] and their findings were later supported by reports of Parveenet et al. [7] and Faisal et al. [8]. Recent reports from our laboratory suggest that this plant extract may have toxicity effect that is specific to the reproductive tissues. Its deleterious effects on total epididymal tissue protein and action on sperm capacitation and acrosome reaction were reported [9].

The role of epididymis in sperm functions such as sperm survival, maturation, motility, capacitation and fertilizing ability cannot be over emphasized. The molecular basis of these functions is beginning to be elucidated in recent time with the discovery of some epididymis-specific proteins and their functions. Some of these proteins include Bin1b (found in different regions of the epididymis) which is important for the acquisition of sperm motility and the initiation of sperm maturation [10], RNase9 (localized mainly in caput), which is important for sperm maturation [11], HongrES1 (found in cauda), which is important in regulation of sperm capacitation and male fertility [12] and Glb1 l4, which is an essential protein in the caput epididymis and is important in epididymal development and sperm maturation in rats [13].

In view of the role of HongrES1 in sperm capacitation [12], the reported action of Q. amara on sperm capacitation [9] has made it imperative to examine the effect of quassin on HongrES1 protein expression.

Materials and Methods

Experimental animals

Adult male Wistar strain albino rats (180-200 g) were housed in well-ventilated animal cages in the Central Animal House, College of Medicine, and University of Ibadan with constant 12-h light 12-h dark cycle. They were fed standard rat feed and clean water ad libitum and were allowed two weeks of acclimatization.

All procedures in this study conformed to the guiding principles for research involving animals as recommended by the Declaration of Helsinki and the Guiding principles in the care and Use of animals [14].

Plant material

The stem bark of Q. Amara was obtained from the Botanical Gardens, University of Ibadan and was authenticated at herbarium of Forestry Research Institute of Nigeria (FRIN), Ibadan, Nigeria. Voucher number 109103 was assigned to the specimen of Q. Amara. The stem bark was air dried and pulverized to afford 2.51 kg of the plant material.

Extraction and separation of quassin

Extraction and fractionation of Q. Amara stem bark were as described by Njar et al. [15]. Briefly, the air-dried stem bark (2.51 kg) was soaked in aqueous methanol at room temperature for 5 days with daily shaking and thereafter was decanted. The extract obtained was concentrated to a dark-green residue (24.4 g) on a rotary evaporator at 40°C. Water was then added and the mixture was extracted with hexane and then with chloroform ($CHCl_3$). The $CHCl_3$ extract were dried with anhydrous magnesium sulphate ($MgSO_4$) and evaporated to give a gum residue. The gum obtained from the extractions were pre-absorbed separately on silica gel and chromatographed on silica gel columns. Elution was done with mixtures of hexane and ethylacetate (Et0Ac) of varying polarity. The fractions eluted with 50% and 60% Et0Ac in hexane were pooled and further purified on preparative t.l.c. using hexane/Et0Ac (3:7 v/v x 2) as the developing agent. A band was scrapped off which upon elution with hexane/Et0Ac (1:9) crystallized from hexane-Et0Ac afforded pure quassin (58 mg, 0.000023% yield). Melting point was 222-224°C. Physical and spectral data were identical with those reported in literature [15]. Monitoring of fraction was done by TLC using Dragendorff reagent, iodine vapour as spray detection and UV lamp.

Experimental design

Quassin was dissolved in ethanol at a dissolution ratio of 1:10,000, a ratio at which the, ethanol has been reported to have no reproductive effect in rats [16]. Fresh quassin was then prepared in distilled water when required and administered daily (orally) for 6 weeks. Three groups of animals of 5 each were used for this study. Group A was the control and received 0.5 ml distilled water (p.o); Group B received 0.1 mg/kg bw quassin while Group C was administered 2 mg/kg bw quassin. At the end of 6 weeks of treatment, all male rats were sacrificed by cervical dislocation. Reproductive and visceral organs were excised, cleared of fat and connective tissue and weighed to the nearest milligram. Tissues and visceral organs for histological studies were preserved in Bouin's solutions while reproductive organs were stored in phosphate buffered saline (PBS, pH 7.4) at -20°C for estimation of total protein.

Another group of animals A*, B* and C* were concurrently treated with 0.5 ml distilled water, 0.1 mg/kg and 2 mg/kg bw quassin respectively for HongrES1 protein expression study. Prior to sacrifice, the rats were sedated by intraperitoneal administration of a combination of ketamine (100 mg/kg bw) and xylazine (10 mg/kg bw). They were then dissected to open the thoracic cavity and expose the heart. Transcardial perfusion was carried out, first with normal saline and thereafter phosphate buffered formalin by passing the fluids through the left ventricle with the aid of a perfusion pump while the right ventricle was nipped to provide for exit. This was to ensure tissues and proteins were fixedin vivo. Animal is fixed when it is stiffened up and fixative discharges through the mouth, ear and nasal orifices. They were thereafter carefully dissected to excise the testes and epididymis and then fixed in phosphate buffered formalin.

Sperm analysis

Epididymal sperm was obtained post mortem by tissue mincing and incubation in PBS. Sperm characteristics analysis was performed on spermatozoa samples collected from the caudal epididymis using Olympus research microscope (Olympus, Japan) under X40 microscope objectives. Progressive motility was assessed immediately. Five-microlitre drop of diluted sperm suspension was placed on a pre-warmed slide and two drops of warm 2.9% sodium citrate was added and covered with cover slip. Progressive forward motility was examined and scored to the nearest 10 [17]. Viability study (percentage of live spermatozoa) was done using eosin/nigrosin stain. The motile (live) sperm cells were unstained while the non-motile (dead) sperms absorbed the stain. The stained and unstained sperm cells were counted and an average value for each was recorded from which percentage viability was calculated. Sperm count was done under the microscope with the aid of the improved Neubauer hemocytometer. Counting was done in five Thoma chambers [18]. The epididymis was immersed in 5 ml normal saline in a measuring cylinder and the volume displaced was taken as the volume of the epididymis [19].

Sperm morphology was evaluated by staining the sperm smears on microscope slides with two drops of Walls and Ewa stain after they were air-dried. The slides were examined under the microscope under oil immersionwith X100 objectives. The abnormal sperm cells were counted and the percentage calculated according to the method described by Wyrobek and Bruce [20].

Estimation of total tissue protein

Total protein was estimated spectrophotometrically according to the method described by Lowry et al. [21] using PBS as standard. Briefly, epididymis and testes were washed in ice cold 1. 15% KCl solution blotted with filter paper and weighed. They were then chopped into bits and homogenized in four volumes of the homogenizing buffer (PBS, pH 7.4) using Teflon homogenizer. The resulting homogenate was centrifuged at 10,000 rpm for 10 mins in a cold centrifuge (4°C) to obtain post mitochondrion fraction. The supernatant was collected and total protein estimated using Randox total protein kit (Randox Laboratories Ltd, United Kingdom).

Hormone assay

An enzyme–based immunoassay (EIA) system was used to measure FSH, LH and testosterone levels in serum samples collected. The EIA kit was obtained from immunometrics (London, UK) and contained the respective EIA enzyme label, EIA substrate reagent and EIA quality control sample. A quality control was carried out at the beginning and at the end of the assay to ascertain the acceptability with respect to bias and within batch variation. Biochemical assays: The serum levels of alkaline phosphatase (ALP), alanine aminotrasferases (ALT) and aspartate aminotransferases (AST) were assayed by the method of Moss and Henderson [22]. Total cholesterol, triglyceride, High-density Lipoproteins (HDL) and low-density lipoprotein (LDL) were measured as described by Rifai et al. [23]. All the biochemical parameters were assayed using the respective commercial diagnostic kits obtained from Diasys Diagnostic systems (Istanbul, Turkey) on a Statfax Diasys 1904 plus Biochemical Analyzer.

Immunohistochemistry technique

Epidydimis and testes were processed for immunohistochemimistry based on methods described by Todorich et al. [24]. Briefly, prepared paraffin embedded tissue slides were deparafinized and rehydrated by immersion in xylene and ethanol. Antigen retrievalwas by microwave heatingin 10mM citrate buffer (pH 6.0) for 20 minutes and subsequent endogenous peroxidase quenching in H_2O_2 and methanol with gentle agitation in Phosphate Buffered Saline (PBS) solution. Thereafter, tissues were blocked for one hour in 5% normal horse serum (NHS) (Jackson Immuno Research lab. Incorporated, USA), then incubated overnight at room temperature in 200 µl of diluted HongrES1 antibody (1:10,000) in a humidity chamber. HongrES1 used was a goat anti-rabbit antibody, obtained from Laboratory for reproduction, Shanghai, China. Dilution was done with 1% of 10% Triton-X and 2% NHS.

Detection of bound antibody was done using appropriate Horse Radish Peroxidase (HRP)-conjugated secondary antibodies in VECTASTAIN ABC kit (Vector Labs) according to manufacturer's protocol. Reaction product was enhanced with DAB for 5-10 minutes, with subsequent dehydration in ethanol and mounting on salinized slides. The immunoreactive tissues were studied and images were acquired with a Sony® digital camera.

Statistical Analyses

Data were expressed as mean ± Standard Error of Mean (SEM). The test of significance between two groups was estimated by student's t-test. $P<0.05$ was considered significant.

Results

Quassin treatment had non-significant effect on the weights of visceral organs viz. lungs, liver, heart, kidney and spleen (Table 1). However, the weight of the testes, epididymis, caudal epididymis, seminal vescicle and prostate gland was significantly lower ($P<0.05$) in the treated groups, when compared with the control (Table 2). Histopathological examination of these organs revealed that the epididymis of treated rats was made up of coiled and hyperplastic tubules filled with ductular eosinophilic material. The ductular epithelium however remained normal (Figure 1). No visible lesion was observed in the testes, but appeared to contain fewer spermatids (Figure 2). Examination of the visceral organs (lungs, liver, heart and kidney) showed that treatment had no effect on the histology of these organs (photomicrograph not shown).

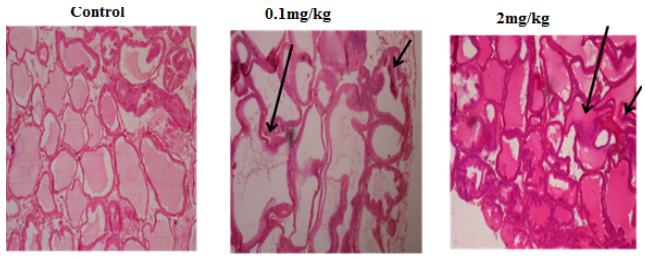

Figure 1: Transverse sections through the epididymis of normal control rats and Quassin treated rats. Arrow indicates coiled and hyperplastic tubules filled with eosinophilic material (X100).

Organ	Lungs (g)	Liver (g)	Heart (g)	Kidney (g)	Spleen (g)
Control	1.48 ± 0.04	6.54 ± 1. 31	0.6 ± 0.09	0.68 ± 0.05	1.01 ± 0.05
0.1mg/kg	1.67 ± 0.08	6.79 ± 0.37	0.68 ± 0.07	0.67 ± 0.03	0.99 ± 0.07
2mg/kg	1.54 ± 0.08	6.12 ± 0.26	0.64 ± 0.02	0.64 ± 0.02	0.93 ± 0.08

Table 1: Visceral organ weights (g) of experimental rats treated with Quassin and corresponding control group.

Values are Mean ± SEM, n = 5, g = gram. Comparisons made with the values of the corresponding control group (student's t-test) showed no significant changes.

Group	Testes (g)	Epididymis (g)	Caudal Epididymis (g)	Seminal Vesicle (g)	Prostate (g)
Control	1.18 ± 0.02	0.48 ± 0.02	0.30 ± 0	1.6 ± 0	0.29 ± 0.02

0.1mg/kg	0.81 ± 0.15*	0.32 ± 0.04**	0.12 ± 0.02**	0.21 ± 0.09**	0.16 ± 0.02*
2mg/kg	0.99 ± 0.14*	0.42 ± 0.01*	0.14 ± 0.02**	0.28 ± 0.09**	0.16 ± 0.01**

Table 2: Effects of Quassin on weight (g) of sex and accessory sex organs.

Mean ± SEM, n = 5, g = gram, *P<0.05, **P<0.001 indicates significant difference from control

Most of the sperm of the control rats had normal counts, motility and morphology. Caudal epididymal sperm parameters showed evidence of toxicity when either treated groups was compared with the control (Table 3). The sperm motility, viability and count were significantly reduced (P<0.05) in the treated groups. Also, the semen volume was significantly reduced (P<0.05) and percentage of sperm cells with abnormal morphology was higher (P<0.05). The apparent reduction in sperm viability and semen volume in the rats treated with 2 mg/kg bw quassin was however non-significant.

Group	Motility (%)	Viability (%)	Count (million/ml)	Volume(ml)	Abnormal Morphology (%)
Control	94.0 ± 1.0	97.25 ± 0.75	121.2 ± 4.24	5.20 ± 0	10.43 ± 0.27
0.1mg/kg	68.0 ± 3.74*	88.6 ± 3.39*	94 ± 7.89 *	5.14± 0.02*	12.43 ± 0.33*
2mg/kg	68.0 ± 3.74*	94.2 ± 1.8	88.8 ± 5.61*	5.16 ± 0.02	12.84 ± 0.66*

Table 3: Effects of Quassin on Sperm parameters.

Mean ± SEM, n = 5, * P<0.05 indicates significant difference from control

Table 4 showed that quassin significantly decreased the epididymal total tissue protein (P<0.05) in both treatment groups, but the testicular total tissue protein was higher than the control. Also serum levels of FSH, LH and testosterone were significantly lower (P<0.05) in the treated groups. Biochemical parameters to assess the serum lipid profile, ALT and AST (Table 5), as well as total serum protein and bilirubin (Table 6) were not affected by quassin treatment. Serum ALP and abumin were however significantly decreased.

Group	Epididymal Total Protein (g/dl)	Testicular Total Protein (g/dl)	Follicle Stimulating Hormone (mIU/mL)	Luteinizing Hormone (mIU/mL)	Testosterone (ng/mL)
Control	1.65 ± 0.32	0.88 ± 0.14	4.42 ± 0.47	1.5 ± 0.18	0.32 ± 0.09
0.1mg/kg	0.60 ± 0.07*	1.26 ± 0.10*	1.6 ± 0.19**	0.72 ± 0.13*	0.09 ± 0.03*
2mg/kg	0.70 ± 0.17*	1.21 ± 0.17*	2.2 ± 02*	0.53 ± 0.17*	0.10 ± 0.03*

Table 4: Effect of Quassin on Tissue total protein and Sex hormones.

Values are Mean ± SEM, n = 5, * P<0.05, ** P<0.01 indicates significant difference from control

Group	Total Cholesterol (mmol/l)	Triglyce ride (mmol/l)	Low Density Lipo protein (mmol/l)	High Density Lipo protein (mmol/l)	Alkaline Phosphatase (U/L)	Alanine Aminotransferase (U/L)	Aspartate Aminotransferase (U/L)
Control	1.38 ± 0.14	0.46 ± 0.13	0.70 ± 0.07	0.32 ± 0.08	144.6 ± 19.9	11.8 ± 3.77	14.0 ± 1.76
0.1mg/kg	1.52 ± 0.1	0.75 ± 0.16	0.87 ± 0.02	0.30 ± 0.03	36.2 ± 2.67**	21.8 ± 5.8	42.6 ± 19.21
2mg/kg	1.32 ± 0.13	0.76 ± 0.13	0.86 ± 0.08	0.24 ± 0.02	20.0 ± 1.64**	19.8 ± 1.24	22.2 ± 5.84

Table 5: Serum Lipid Profile and Liver Function Biomarkers of rats treated with Quassin

Values are Mean ± SEM, n = 5. ** P<0.001, indicates significant difference from control

Group	Total Bilirubin	Unconjugated Bilirubin	Serum Total protein	Serum albumin
Control	4.8 ± 0.58	0.34 ± 0.05	37.6 ± 0.81	23.6 ± 0.93
0.1mg/kg	3.8 ± 0.37	0.28 ± 0.09	35.4 ± 0.75	23.2 ± 0.8
2mg/kg	5 ± 0.71	0.3 ± 0.07	35.6 ± 1.47	18 ± 0.55**

Table 6: Serum Protein and Bilirubin of rats treated with Quassin.

Values are Mean ± SEM, n = 5. ** P<0.001, indicates significant difference from control

Immunohistochemical studies show a great expression of HongrES1 protein in the epididymis of the control rats. This protein expression was greatly suppressed by quassin treatment. Figure 3 shows a decline in staining intensity of the epididymal cells when the control was compared with the treated groups.

Lack of brownish coloration after immunohistochemical staining of the testes was indicative that HongrES1 was not expressed in the rat testes (Figure 4).

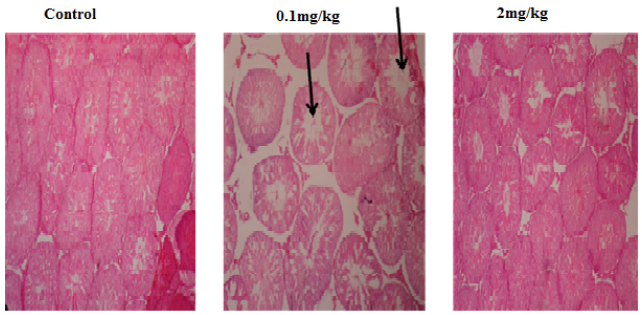

Figure 2: Transverse sections through the testes of normal control rats and Quassin treated rats. No visible lesion was observed, but low dose group contain fewer spermatids (X100).

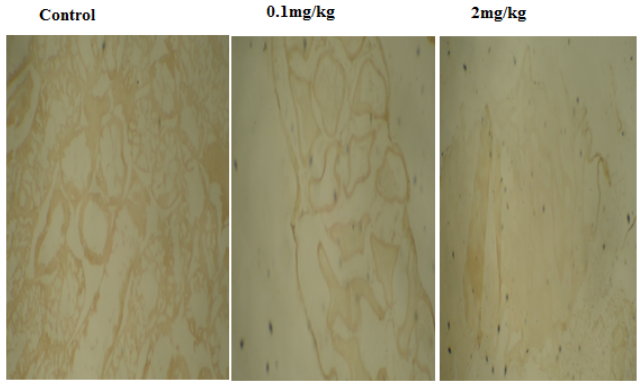

Figure 3: Patterns of expression of HongrES1 protein in caudal epididymis of rats treated with Quassin

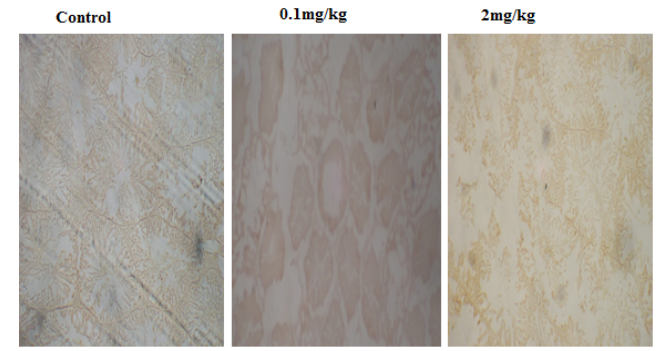

Figure 4: Lack of expression of *HongrES1* protein in testes of rats treated with Quassin

Discussion and Conclusion

Earlier reports from our laboratory showed that methanol extract of Quassia amara stem bark interfered with the structural integrity and functional competence of sperm cells in vivo [9]. Quassin, the major bioactive constituent of Q. Amara was shown in this study to replicate these effects. A decrease in organ weight restricted to the reproductive tissues was observed after quassin administration. Sperm motility, count and morphology significantly declined with quassin treatment. Quassin (0.1 mg/kg) appears to have a greater effect on these organ weights and sperm characteristics than 2 mg/kg quassin. Sperm viability and semen volume were decreased by 0.1 mg/kg quassin treatment. Serum levels of FSH, LH and testosterone were also observed to decrease in both doses of treated group. Raji and Bolarinwa [1] earlier reported a similar outcome using the plant stem wood. Since testosterone is important for initiation and maintenance of spermatogenesis, it is plausible that decline in weight of the reproductive tissues after quassin treatment is due to a combination of decreased hormone level and a decline in sperm count [25]. Raji and Bolarinwa [1] reported a decrease in weight of the anterior pituitary gland and a decline in in-vitro testosterone production from isolated Leydig cells when intact rats were treated with Q. Amara extract, and they hypothesized that Q. Amara site of action may include the pituitary gland and the Leydig cells.

The contributory factors to the initiation of spermatozoa motility and maturation, mainly in the form of proteins and small molecular weight glycoproteins emanate from the epididymal epithelia cells [13]. Derangement of the epididymal tubules and impairment of motility of caudal epididymal sperm of treated rats is a reflection of the effect of quassin on the functional anatomy of the epididymis. Therefore, quassin may mediate its spermatoxic action by acting on epididymis.

The decrease in epididymal tissue total protein recorded in the treated group further emphasizes the toxicity effect of quassin on the epididymis. We earlier reported that Q. Amara treatment declined chances of successful sperm capacitation and probably make them fertilization incompetent [9]. Thus, epididymis may be the most probable site of action. Androgen decline may not be the mediator for reproductive toxicity of quassin, as Parveen et al. [7] reported that Q. Amara had no effect on biomarkers of prostate and seminal vesicle functions, which are also androgen dependent. However, the observed decrease in sperm count and the increased number of morphologically abnormal sperm suggests interference with testicular spermatogenesis.

Lack of effect on sperm viability (2 mg/kg quassin) in vivo and sperm motility in vitro (data not shown) further suggests reproductive effect probably not due to cytotoxicity or direct toxicity. Action of quassin on epididymis and epididymal tissue total protein therefore calls for observation on protein expressions in this organ.

HongrES1 expression, an epididymal protein that is specifically expressed in the caudal epididymis of rats, humans and other mammals [26] and shown to critically time sperm capacitation and acrosome reaction [12] was observed in this study. Decline in HongrES1 expression observed in quassin treated rats suggests that quassin may be inducing infertility probably by suppressing HongrES1 expression and therefore repressing chances of successful sperm capacitation.

Save for the observed decline in serum albumin (2 mg/kg bw), lack of effect of treatment on visceral organ weights, serum lipid profile, liver function biomarkers, serum protein and bilirubin levels (Tables 1,5 and 6) are consistent with a specific effect of quassin on reproductive system, without systemic toxicity at doses administered [27]. Parveenet et al. [7] earlier reported that Q. Amara extract had no effect on hematological and serological parameters. The observed decline in serum ALP may be inferred to mean quassin may be hepatoprotective at doses administered.

Action on proteomics may therefore explain mechanism of action of quassin. Thus decline in sperm parameters observed may be via action on proteins or androgens or both. The reason for observed increase in testicular total tissue protein is unclear, but may be due to a feedback response to insults from quassin treatment.

Conclusion

Evidence of toxicity specific to reproductive tissues, especially the epididymis, coupled with down regulation of epididymal protein HongrES1 after treatment of male rats with quassin suggests that quassin may be a potential candidate for male contraception.

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