Original Article

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Adverse Effects of Hydroxychloroquine on Testicular Tissue and Sperm Quality: An Experimental Study on Male Albino Rats

Physiology Section

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ABSTRACT

Introduction: Hydroxychloroquine (HCQ) is a medicine that has mainly been used to treat malaria. During the recent Coronavirus Disease-2019 (COVID-19) period, an abrupt spike in the sale of HCQ has been found. The repurposing of this drug in the treatment of COVID-19 was not only found to be ineffective but also showed many side effects. The adverse effects of the enormous exposure to this drug should be unveiled.

Aim: To evaluate the acute effect of HCQ exposure on the male reproductive system by analysing the changes in antioxidant enzyme levels in testicular tissue, sperm quality, and histopathological assessment of testicular tissue using male albino rats as the subject model.

Materials and Methods: The present experimental study was done by using male albino rats as the subject model. It was conducted in the laboratory of the Department of Human Physiology at Raja NL Khan Women's College (Autonomous) in Medinipur, West Bengal, Kolkata, India, from the last week of June 2023 to the first week of August 2023. A total 10 male albino rats were obtained and randomly divided into two groups (n=5 in each group), control and HCQ-treated, and were given plain drinking water and 33 mg/kg/day HCQ, respectively, for six consecutive days through oral gavage. After the treatment tenures, rats were euthanised, and antioxidant enzymatic activity was measured by a spectrophotometer. Sperm quality and testicular histopathology were studied. A two-tailed t-test was used to compare the parameters of the control and HCQ-treated groups.

Results: The total testicular weight of the control group was 2.37±0.17 g, in contrast treated group had a significantly lower testicular weight of 1.98±0.19 g. A significant decrease in total testicular protein in the treated group was found, i.e., 28.86±3.03 mg/g of tissue, compared to the control group, i.e., 60.46±2.85 mg/g of tissue. The sperm count of the control group was 4.51±0.06 million/ml of suspension, while the treated group had a significantly lower sperm count, i.e., 2.73±0.11 million/mL of suspension. The percentages of sperm motility and viability in the control group were 86±1.63% and 94±1.33%, respectively, but in the treated group, the percentages were significantly lower, i.e., 22.66±1.35% and 24±1.78%, respectively. The increasing sperm anomalies after HCQ exposure indicate poor sperm quality.

Conclusion: Infertility or reduced fertility can result from testicular dysfunction, in continuation, decrease in sperm count, motility, viability, together with abnormalities in sperm morphology, was observed after this exposure. Thus, the present study provides insight into the adverse effects of the use of this drug, which might be associated with male infertility.

Keywords: Antioxidant enzyme, Coronavirus disease 2019, Histopathology, Sperm count, Testicular toxicity

INTRODUCTION

The use of HCQ in COVID-19 clinical trials was tremendous. In 2020, the sales volume of HCQ showed a high peak in India [1]. HCQ is an antimalarial drug, and its mechanism is related to the detoxification process of plasmodium parasites, but the mechanism against malaria is not completely clarified [2]. A dose of HCQ at 33 mg/kg/day for six days might be associated with the impairment of kidney and liver tissues [3]. According to Centre for Disease Control (CDC) guidelines, the normal dose for acute malaria is 800 mg followed by 400 mg at 6, 24, and 48 hours [4]. For malarial prophylaxis, the considered dose is 400 mg once weekly on the same day of the week, starting two weeks before visiting a malaria-prone region and continuing for four weeks after leaving the region, as stated by the Mayo Foundation for Medical Education and Research (MFMER) [5]. In contrast, during the COVID-19 period, HCQ was used at a dose of 200 mg×2/day for 5 days [6] and even 200 mg×3/day for 10 days [7].

The reproductive system is the most important part of the entire physiological system, and damages in the reproductive part can be fatal. Causes of male infertility are frequently investigated by many researchers, but the exact reasons are not well established. Exposure to chemical agents such as pesticides, hormonal agents, therapeutic drugs, including antibiotics, may lead to male infertility. Oxidative stress in testicular tissues also plays a leading role in damaging the male reproductive system. Antioxidant enzymes such as Superoxide Dismutase (SOD) and Glutathione-S-Transferase (GST) present in testicular tissue fight against the generation of Reactive Oxygen Species (ROS), but disparity in these antioxidant enzymes can lead to the formation of oxidative stress. Research has showed that antibiotics can change the levels of these antioxidant enzymes and are associated with the disturbance of cellular redox status by increasing the production of ROS [8].

Pharmacovigilance of this drug on reproductive parameters is urgently needed for further awareness. Thus, this study aimed to examine the effect of HCQ on the male reproductive system by analysing the oxidative stress markers in the testis, studying the morphology and quality of sperm, and assessing the histopathology of the testis.

MATERIALS AND METHODS

Ten male eight-week-old albino rats weighing about 150-160 g were obtained from Saha Enterprise, Kolkata, West Bengal, India, via local suppliers and housed in a standard climate-controlled facility with a regular day-night cycle. The rats were acclimatised for seven days. HCQ sulfate was purchased from Sigma-Aldrich. The animals were randomly divided into two groups, each containing five animals - control and HCQ-treated groups. All groups received the same volume of drinking water through oral gavage from the 8th day until the completion of the treatment tenures. Treated group received HCQ sulfate at a dose of 33 mg/kg/day through gavage

with drinking water for six days, considering the lethal dose of 1240 mg/kg body weight of a rat [9,10]. In present study, a single dose was chosen within a range reflecting typical doses for malaria treatment and COVID-19 treatment in humans, as mentioned in the introduction section. This dose was then converted to an equivalent dose for rats following Food and Drug Administration (FDA) dose conversion guidelines [11]. Moreover, the treatment tenure was also selected on the basis of COVID-19 treatment tenures as mentioned earlier. The body weight of each rat was recorded daily, and the doses were adjusted day by day. Oral gavage was given to justify the exact amounts of the given doses.

The present experimental study was done by using male albino rats as the subject model. It was conducted in the laboratory of the Department of Physiology at Raja NL Khan Women's College (Autonomous) in Medinipur, West Bengal, Kolkata, India, from the last week of June 2023 to the first week of August 2023. Animal experiments were performed with the approval of the Institutional Animal Ethics Committee in accordance with national guidelines {Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA)}. The ethical approval number is 04/IAEC (1)/S/RNLKWC/2023.

Inclusion criteria: Only healthy male rats of eight weeks of age are selected for present study. No rats were experimented on before the completion of the acclimatisation period. All experiments began after obtaining ethical approval.

Exclusion criteria: Diseased or unhealthy rats were excluded from the study.

Study Procedure

The HCQ was given through oral gavage using drinking water as a vehicle. The control group also received an equal volume of drinking water through gavage to nullify the stress that might be generated for gavaging. HCQ was given at 12 pm daily to avoid the occurrence of any stress due to periodic changes. The rats were accommodated in a proper day-night cycle and climate-controlled facility to exclude any stress from environmental consequences.

After completing the total treatment tenures, the rats were euthanised, and the following study parameters were done.

Tissue weighing, preparation, and total protein quantification: After each treatment tenures, testes and cauda epididymis were excised after euthanisation of each rat. The total weight (g) of both testes was measured. The excised testes were homogenised in ice-cold isotonic sodium phosphate buffer {Phosphate Buffered Saline (PBS)} (0.2M, pH 7.4) and centrifuged for 30 minutes at 10,000 g at 4°C. Supernatants were collected, and total proteins were estimated by Biuret method [12].

Following enzymatic quantifications were further done:

Measurement of antioxidant enzymatic activity: Determination of Superoxide Dismutase (SOD) activity: SOD was quantified by the method described by Beauchamp C and Fridovich I, (1971) [13]. The optical density was measured at 560 nm, and the activity was expressed in µmol/min/mg of testicular protein.

Determination of Catalase (CAT) activity: CAT activity was determined from the collected supernatants according to the method described by Aebi (1984) [14]. The optical density was measured at 240 nm for 60 sec using a spectrophotometer. The activity was determined by using a molar extinction coefficient of 39.4 M-1 cm-1 and expressed in µmols/min/mg of testicular protein.

Determination of Glutathione Reductase (GR) enzymatic activity: GR activity was determined from the collected supernatants by the method described by Carlberg I and Mannervik B (1985) with a molar coefficient of 6.22 mM-1 cm-1 [15]. The optical density was measured for three minutes at 340 nm, and the activity was expressed in nmol/min/mg of testicular protein.

Estimation of Glucose 6 Phosphate Dehydrogenase (G6PD) enzymatic activity: G6PD activity was determined from the collected supernatant according to the method described by Beutler E (1975) using a molar coefficient of 6.22 mM-1 cm-1 [16]. Optical density was measured for three minutes at 340 nm. The activity was expressed in nmol/min/mg of testicular protein.

Estimation of Glutathione S Transferase (GST) activity: GST activity was done according to the modified method of Mannervik B (1985) [17]. Optical density was measured at 340 nm for five minutes using a molar coefficient of 0.0096 µM-1 cm-1. The activity was expressed in nmol/min/mg of protein.

Lipid peroxidation assay: Lipid peroxidation was determined following the method described by Ohkawa H et al., (1979) [18]. The optical density of Malondialdehyde (MDA) was determined from the collected supernatant by spectrophotometric analysis at a wavelength of 532 nm with a molar coefficient of 1.56×105 M-1 cm-1.

Sperm suspension preparation and its quality assessment: Sperm were collected from the cauda epididymis and suspended in PBS (140 mM Sodium Chloride (NaCl), 10 mM phosphate buffer, pH 7.2). Viability, motility, and morphological analysis were conducted according to Karaman M et al., (2018) [19]. Viability was determined by staining with eosin-nigrosine at a 1:2 ratio of sperm suspension and stain, where pink stained sperm were considered dead and unstained ones were living which was measured per 100 total sperms. Motility percentage was calculated by considering the sperm to be able to move in a direct line. Sperm morphology was determined by counting abnormal-shaped sperm at both 100x and 400x magnification. Visualisation of these parameters was done under a light microscope at 100x magnification. Total sperm count was done using a hemocytometer after placing the sperm suspension. Sperm count was conducted on four fields using a light microscope at 100x magnification.

Histopathological analysis: Testicular tissue sections of 5 µm thickness were prepared, and histopathological analysis was done under 100x and 400x microscopic magnification using the Haematoxylin and Eosin (H&E) staining method.

STATISTICAL ANALYSIS

Data were calculated using a two-tailed t-test, and the parameters of the control group were compared with the HCQ-treated group, considering significance at $p<0.05$, highly significant at $p<0.01$, and extremely significant at $p<0.00001$. Results were expressed as mean and standard deviation. Statistical analysis was done by using Statistical Packages for the Social Sciences (SPSS) {International Business Machine (IBM) SPSS 29.0.2.0}.

RESULTS

Testicular Weight and Total Protein Level

The total testicular weight (g) significantly decreased in the treated rats, measuring 1.98±0.19 g, compared to the control group, which was 2.37±0.17 g. Evaluation of testicular protein (mg/g of tissue) showed that the total protein value in the control group was 60.46±2.85 mg/g of tissue, in contrast HCQ-treated rats had a protein value of 28.86±3.03 mg/g of tissue. This shows extremely significant difference in the testicular protein value between the control and treated rats. The decrease in total protein value after HCQ exposure indicates tissue damage.

Evaluation of Oxidative Stress (OS) Markers

After analysing the activity of different antioxidant enzymes, we found that the CAT, SOD, GR, G6PD, and GST activity in HCQ-treated rats significantly decreased than that in the testicular tissue of the control group. Additionally, the MDA level was seen to increase in the treated rats compared to the control group, as shown in [Table/Fig-1].

Oxidative stress markers	Values of control group (mean±SD)	Values of HCQ treated group (mean±SD)
SOD activity (µmol/min/mg of protein)	8.17±0.63	3.24±0.89**
CAT activity (µmol/min/mg of protein)	2.92±0.35	1.73±0.44**
GR activity (nmol/min/mg of protein)	9.83±0.53	6.63±0.76**
G6PD activity (nmol/min/mg of protein)	5.83±0.92	2.81±0.29**
GST activity (nmol/min/mg of protein)	64.2±3.49	48.85±6.85**
MDA level (nmol/mg of protein)	4.65±0.36	20.59±2.27***

[Table/Fig-1]: Antioxidant enzymatic activity and MDA levels in testicular tissues of control and HCQ treated rats.
Data are expressed as mean±SD of n=5 rats per group. Statistically significant difference expressed as ***p<0.00001 and **p<0.01, for comparing the control and treated group
SOD: Superoxide dismutase; CAT: Catalase; GR: Glutathione reductase; G6PD: Glucose 6 phosphate dehydrogenase; GST: Glutathione-S-transferase; MDA: Malondialdehyde

Sperm Parameters

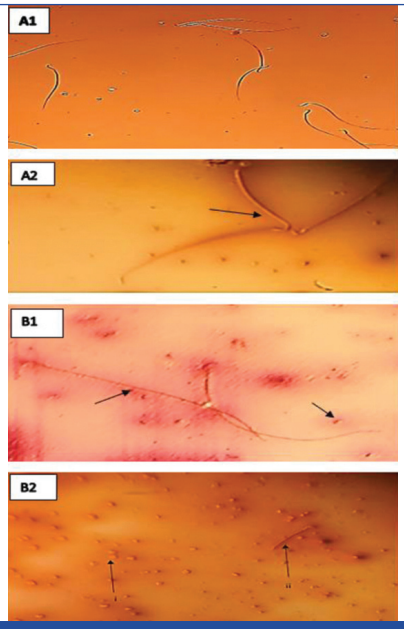
The sperm count was found to be significantly decreased in rats exposed to HCQ compared to the control group. Sperm motility and viability were also observed to be lower, and there were more instances of broken head and tail anomalies in the treated rats than in the control group, as shown in [Table/Fig-2].

Sperm parameters	Control group (mean±SD)	HCQ treated group (mean±SD)
Sperm count (million of cells/mL of suspension)	4.51±0.06	2.73±0.11***
Sperm motility %	86±1.63	22.66±1.35***
Sperm viability %	94±1.33	24±1.78***
Broken tail anomalies %	19.2±3.6	63±3.31***
Broken head anomalies %	6.2±2.32	12.2±2.04**

[Table/Fig-2]: The effect of HCQ on sperm parameters. Data are expressed as mean±SD with n=5 animals per group.
Statistically significant difference in comparing of control with HCQ treated group (**p<0.01, ***p<0.00001)

Photomicrographs of Sperm Morphometry

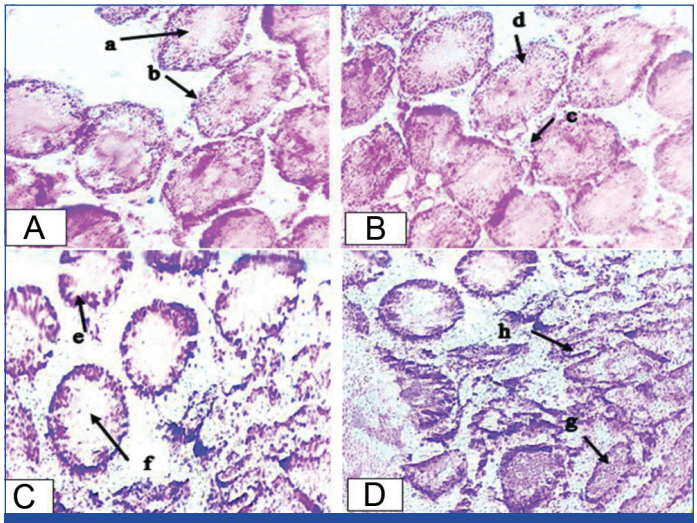
More live or unstained sperm were found in the control group than in the treated group. In the treated group, most of the sperm were stained, indicating an increased number of dead sperm compared to the control group. Furthermore, the treated group exhibited a higher number of head and tail anomalies in the sperm, as shown in [Table/Fig-3].



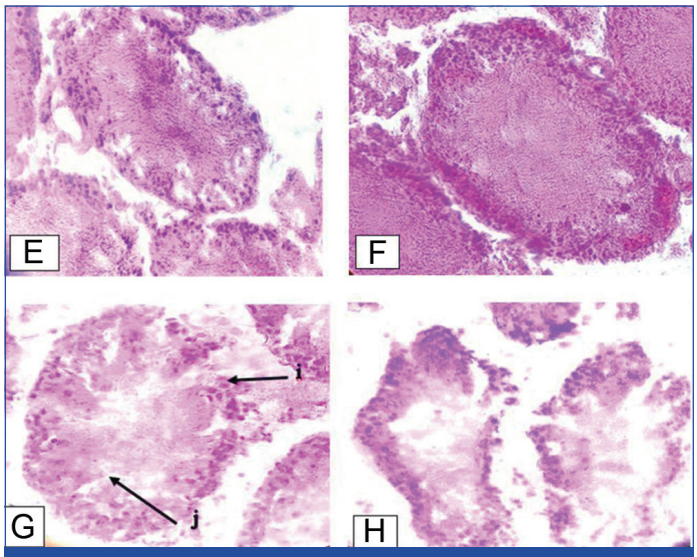
[Table/Fig-3]: A1. Sperm of control group (magnification 100x) showing the content of live sperms without anomalies; A2. Sperms of control rats showing the prominent head and tail structure and unstained (magnification 400x); B1. Sperm of HCQ treated rats (magnification 100x) showing the sperm anomalies (arrow showing broken tail and stained dead sperms) and lower sperm in one microscopic field; B2. (i) Only heads of the sperms present tails are broken, (ii) broken tip of the tail (magnification 400x).

Histopathological Assessment of Testis

After histopathological assessment, altered testicular histology was found in the HCQ-treated group. Ruptured germinal epithelium and decreased spermatogonia cells were seen in case of treated rats. Outward protrusion of Sertoli cells due to the rupture and shrinkage of seminiferous tubules was seen in the treated group. In contrast, the control group showed normal testicular histology, as shown in [Table/Fig-4] at 100x and [Table/Fig-5] at 400x microscopic magnification, respectively.



[Table/Fig-4]: Microscopic structure of seminiferous tubules of control and treated rats (H&E 100x). A) Seminiferous tubules of control rats with: a) Proper seminiferous lumen, b) Prominent germinal epithelium with intact Sertoli cells; B) Testicular histology of control rats, c) Presence of Leydig cells, d) Proper spermatogonia cells are present; C) Histological alteration in HCQ-treated rats, e) Ruptured germinal epithelium, f) Decreased spermatogonia cells; D) Testicular histology of HCQ-treated rats, g) Shrinkage of seminiferous tubule and no lumen space observed, h) Ruptured seminiferous tubules.



[Table/Fig-5]: Microscopic structure of seminiferous tubules of control and treated rats (H&E 400x). E&F) Proper and large structure of seminiferous tubule with intact germinal epithelium and sertoli cells of control rats; G) seminiferous tubule of HCQ-treated rats with i) Ruptured germinal epithelium and outward protrusion of sertoli cells; j) Haemorrhages and patchy structrures insde the lumen; H) Shrinked seminiferous tubules.

DISCUSSION

Excessive use of medicines leads to many health problems all over the world. Effects of various medications on the male reproductive system have been well established, like amoxicillin, gentamicin, and cefazolin. An increase in oxidative stress was also demonstrated in testicular tissue by these medications. However, until now, the side effects of HCQ on testicular tissues have been unknown. The adverse effects of HCQ on the liver and kidney have been studied by evaluating the increased levels of liver and kidney enzymes after HCQ exposure [3].

In this study, after six days of HCQ exposure, total weight of testes and testicular protein decreased. HCQ is a synthetic analogue of chloroquine, and chloroquine has been found to be more dangerous than its analogue. Ibrahim S et al., in their study, found occurrences of orchidotoxicity due to chloroquine exposure, but they did not find a significant adverse effect due to HCQ exposure. Yet, they also mentioned that differences in results may occur due to variations in animal models, age groups, and drug exposure periods in other result [20]. The present study focuses on the acute effects of HCQ exposure. A significant decrease in total testicular protein and testis weight was observed, indicating that chloroquine-like testicular damage might occur due to HCQ exposure as well.

Antioxidant enzymatic activities such as SOD, catalase, GR, G6PD, and GST also decreased, while MDA levels increased after HCQ exposure compared to the control. This signifies the occurrence of oxidative stress in testicular tissue after HCQ exposure. In a previous study, Uzar E et al., found that oxidative damage might occur in some vital organs due to HCQ exposure [21]. In another study, Atli O et al., reported that azithromycin leads to decreased SOD and CAT levels in cardiac tissues, together with an increase in MDA levels upon azithromycin exposure [22].

In present study, sperm count, sperm motility, and viability were found to be significantly lower after exposure to the tested medication. Conversely, there was a significant increase in the percentage of anomalies in sperm morphology in the treated rats compared to the control group.

Samplaski and Nangia AV, have also reported that the use of common medications can lead to alterations in sexual function, like decrease in semen volume and changes in sperm parameters [23]. Various antibiotics including amoxicillin, ofloxacin, ciprofloxacin hydrochloride, nitrofurantoin monohydrate, doxycycline hyclate, and cefuroxime axetil were tested on cryo-preserved-thawed sperm. It was found that ciprofloxacin affects sperm hyperactivation by altering the sperm membrane, while ofloxacin improved sperm fertilisation capacity. Nitrofurantoin decreased sperm motility and fertilisation capacity, cefuroxime affected sperm motility, amoxicillin affected motility, and doxycycline affected the sperm capacitation process. Further investigations was done on the effects of various antibiotics such as co-trimoxazole, erythromycin, amoxicillin, tetracycline, and chloroquine. As per Luo M et al., 2022 tetracycline at a concentration as low as 2.5 mg/mL can inhibit sperm motility [24]. High doses of nitrofurantoin can halt the development of the testicles due to the incapacity of testicular cells to utilise oxygen and carbohydrates according to Pergialiotis V et al., 2018 [25].

The present work found alterations in the histology of the testes after HCQ exposure compared to the control group. Visual decreases in the diameter of seminiferous tubules due to HCQ exposure indicate impaired testicular function. The germinal epithelium was found to be ruptured in the treated group compared to the control. Decreased spermatogonia cells, presence of haemorrhage in the lumen, and shrinkage of seminiferous tubules suggest potential testicular atrophy or impaired spermatogenesis. In a recent histological study of testes after HCQ treatment, Alruwaili M et al., found that there was spermatid sloughing, testicular interstitial oedema, and spermatogenic arrest that might occur due to chronic HCQ exposure in male albino rats [26]. In a study by Kheirandish R et al., it was found that seminiferous tubules were damaged and sperm quality was poor after ciprofloxacin exposure [27].

Limitation(s)

The report of present work is based on a sample size of 10 animals due to ethical considerations and guidelines that limit the use of animals. The present study was conducted to investigate the possible acute adverse effects on testicular parameters due to HCQ exposure. Further research with a larger sample size is recommended. Though, the present study provides insight into the adverse effects of HCQ on the male reproductive system, additional studies required on the female reproductive system to potentiate the knowledge regarding the toxicological effects on the reproductive system. Furthermore, remedial studies are necessary to prevent the damages that come with this drug.

CONCLUSION(S)

Although HCQ has been used to treat different kind of diseases, its use peaked during the COVID-19 period. The continuous increase in the use of this drug may potentiate risks to human health. Thus, present findings mainly focus on the acute exposure to HCQ, which may affect the male reproductive system. The decreased activity of antioxidant enzymes indicates the generation of Reactive Oxygen Species (ROS), which may have led to damage in the male reproductive system by inducing oxidative stress on the testicular tissues. The decreased sperm count, sperm motility, and sperm viability, together with increased structural anomalies, indicate damage to the male reproductive system. The rupturing of seminiferous tubules and alterations in the histology of the testes after this exposure indicates the abnormal changes in the reproductive organs. Overall, it can be inferred that unnecessary exposure to HCQ may be associated with the incidence of male infertility.

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