

Cytotoxic effect of Quinidine on testicular tissues, sperm parameters and Ca^{2+} level in mice sperms; as an option of male contraception

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Abstract

Recently, there is developing of a new approach push of male contraception, based on targeting specific processes in sperm development, maturation, and function by using quinidine as a one of calcium channel blocker.The effect of antimalarial alkaloids Quinidine (QD) investigated in the testicular tissue, Ca^{2+} level and some sperm parameters in male mice. To evaluate the impact of QD as a Ca^{2+} channel blocker on Ca^{2+} level, the sperms are exposed to QD directly at concentration 10 and 50 µm through perfusion apparatus have a multiple connecting tubes which perfuse QD directly to the imaging chamber containing sperms, then the Ca^{2+} imaging have done by using laser scanning fluorescence microscope. While the effects of QD on the sperm parameters (motility, abnormality and death percentages) observed by incubation sperms in chambers containing QD at 500 µm for 2 hours. The result showed a significant decrease ($p<0.05$) in sperm motility, with an increase in abnormality and death percentages with reversible recovery after Hanks Solution (HS) wash at pH 7.4.On the other hand the sperms were incubated in QD at concentrations 10, 50 µm from 2 to 14 hours respectively, have no any changes on in these sperm parameters and testicular tissues at these lower concentration of QD. Although the effects were apparent on sperm parameters after incubation with QD at 500 µm for 2 hours. Histology disturbance was significantly increased after incubation of testis tissue for 14 hours with QD at Concentration 500µm as compared to control. The changes caused by QD only recovered on sperm parameters and Ca^{2+} level after washing the sperm with Hanks solution medium (HS) medium containing 2mM Ca^{2+} at pH 7.4. So under the exposure to QD, the inhibitory effect on fertility parameters of mouse's sperms are reversible due to Hanks (HS) supplementation, but there is no recovery determined in testicular tissues treated by QD even when it washed by HS supplementation.

Keywords: Male Fertility, Testis, Sperms, Calcium Blockers, Male Contraception

1. Introduction

Quinidine (QD) is a rapidly acting blood schizonticide with a long history as anti-malarial drug, gradually, concerned in reproductive toxicity (Farombi, Ekor et al. 2012). In past, QD used as a cytotoxic induce and aid labor at a last stage of pregnancy (Mukherjee and Bhose 1968) and in contraceptive, even though its spermicidal action described as weak (Trifunac and Bernstein 1982). Other studies confirmed QD as a testicular toxicant through lowers serum and testicular testosterone in adult Sprague-Dawley rats (Osinubi, Ajala et al. 2006). In addition, QD induces spermatogenic epithelial toxicity by interfering with the steroidogenic function of the Leydig cell (Osinubi, Ajala et al. 2006).In general the damage of seminiferous tubular cells and the testicular interstitial cells persisting even after the cessation of QD exposure (Osinubi, Noronha et al. 2006). Recently, biomedical reporters have investigated the toxic effects of these antimalarial alkaloids on human and animal spermatocytes have applied both in vivo animal and in vitro

techniques (Trifunac and Bernstein 1982). The inhibitory effect of QD and on sperm metabolism (measured by production of lactic acid and CO_2) and motility have been investigated (Trifunac and Bernstein 1982) . As well as, Garg et al. (1994) showed QD spermicidal activity in human sperms revealed within 20s of incubation with alkaloid ,100% of human sperms immobilized (Garg, Doncel et al. 1994). In vivo, QD had a toxic effect on the seminiferous tubules of Sprague-Dawley rats administered QD in a short term and may disturb spermatogenesis. In addition, in another vivo study in Sprague-Dawley rats has observed degenerative changes in the germinal epithelium. Also, a significant decrease in sperm count, activity, and morphology percentage (Nwangwa, Igweh et al. 2007) agrees with Abayomi et al. (1992) reported male rats infertility caused by chloroquine treatment with delay in germ cell developmental arrest result in depletion of spermatids (Osinubi, Akinlua et al. 2004). All these effects can suggested the requirement for the investigation that QD can take place as a male contraceptive in humans (Osinubi, Akinlua et al. 2004).Ion channels have a fundamental role in involvement sperm fertility processes: maturation, capacitation and acrosome reaction. Furthermore the blocking of calcium channels by pharmacological inhibitors or compounds from isolated from plant were reported as one of promising mechanisms of future male contraceptives (Driák and Svandová 2012).The sperm channel approach based on inhibition of calcium entry to sperm and, as a result, shows its effected on testicular function and spermatogenesis, which investigated for several decades. This approach achieves enough suppression of spermatogenesis to succeed contraception in most men, but not all

We concluded from this study, the scheme based on findings of other studies in non-human subjects that have short-term uses of agent, to treat malaria have a significant antispermatogenic and antifertility or contraceptive effect on mice sperms and testicular tissue in vitro, and our findings showed the the toxic effect of QD on sperms parameters concluded that QD had harmful effects on seminiferous tubules and sperm formation in male mice and probably caused disorder in spermatogenesis and male fertility , although in vivo has not been investigated by our recent study, suggesting to take more attention in future studies to determine the toxic effect on sperms in vivo experiments and find the specific molecular target blocked by this anti-malarial alkaloids result in inhibition of male fertility.

2. Aim of Study

This option based on suppression of Ca^{2+} entry to sperm, and thus of testicular function and sperms behavior can realize adequate suppression of sperms used for effective contraception in most males but not all. Otherwise, the non-hormonal approach is based on identifying specific processes in sperm development, maturation, and function. A variety of targets identified in animal models and these targeted successfully affected. This mode, nevertheless, still under pre-clinical field at present, thus, basis for bearing in mind should be safe, efficient and reversible approaches for male contraception can be developed.

3. Materials and Methods

3.1 Chemical Reagent

Most of the reagents, Quinidine and Cell-Tak were purchased from Sigma-Aldrich (St. Louis, MO, USA). Flou4-AM and pluronic F-127 were from Molecular Probes (Invitrogen, Eugene). Supplemented Hanks solution (HS) prepared in the laboratory and contained in a Mm concentration of (135 NaCl, 5 KCl, 2 $CaCl_2$, 1$MgCl_2$, 30 HEPES, 10 Glucose, 10 Lactic Acid, 1Na pyrovate, adjusted to PH 7.4 with NaOH).

3.2 Animals

The animal uses followed guidelines approved by AAALAC Intl (Center for Animal Care) at Wuhan University. Experiments involving Wild-type (WT) Age- mice, three months old, were used for sperm collection and the testis culture.

3.3 Sperm Preparation

Mice killed by CO2 asphyxiation, followed by cervical dislocation. The caudal epididymides excised and rinsed with Hanks (HS) medium [9] containing in (millimolar) 135 NaCl, 5 KCl, 2 CaCl2, 1MgCl2, 30 HEPES, 10 Glucose, 10 Lactic Acid, 1Na pyrovate, adjusted to PH 7.4 with NaOH. Released sperm were concentrated to 5× 10 6 to 1×10 7 /ml by centrifugation for five minutes at 500×g. Used for estimation the behaviors including (motility, abnormal shape and counting the dead sperms by incubate the sperms in hanks solution (HS) medium containing the quinidine at concentration 10, 50, 500µm for 2-14 hours.

3.4 Testis Culture

Followed the guideline of (Gohbara, Katagiri et al. 2010). The testes of male mice were removed then

encapsulated, and tissues separated by forceps into 2 to 8 pieces of 2-3mm in diameter. Then tissue fragments were directly placed in Hanks Balanced Salt Solution (HBSS) on ice. In the laboratory, the tunica was removing from the testis and parenchymal tissue was cutting into 3–5mg (2–3mm) pieces placed on the culture plate well. They were then soaked in the culture medium for more than 24 hours to replace the water in them. The culture incubator supplied with 5% carbon dioxide in the air and maintained at 34ºC. Before viewing testicular tissues under confocal microscope, the tissues culture placed in the imaging chamber containing culture medium for the control group and others containing tissue culture containing QD at 10, 50, 500µm for 2-14 hours. After that the samples loaded with fluo4-Am for 30 minutes preparing for imaging followed by two washing in tissue culture medium.

3.5Measurements of Ca^{2+}

Changes in $[Ca^{2+}]i$, was measured by using Ca^{2+} imaging as described in previous studies (Xia, Reigada et al. 2007, Xia and Ren 2009). Sperms loaded with the respective fluorescent Ca^{2+} indicator 4 µm Fluo-4 AM and 0.05% Pluronic F-127 for 30 minutes.At the room temperature in the dark, followed by twice washing by Hanks medium (HS) medium at pH 7.4 to remove excess fluorescence dyes from sperms loaded samples, washed sperm plated onto coverslips coated with Cell-Tak. Small-volume imaging chambers (~1 cm diameter [90 µl]) were formed with formed with Sylgard on coverslips. Waiting 10 min to allow sperm to attach to attach for 10 min. Laser confocal monochromator (UK) with 75-W Xenon lamp was used to generate the excitation at 491nm. 60X objective and 1.63 adaptor on an inverted microscope used for imaging, and collected with the cooled charge-coupled device camera (CoolSNAP HQ; Roper Scientific, Tucson, AZ). In order to, the 300 repeat interval in every five seconds. Data collection and image processing are done using commercial software (Image J software). $[Ca^{2+}]_i$ changes are presented as ΔF: F0 ratios as described in (Nash, Lefievre et al. 2010). After background subtraction, ΔF is the percentage change in intensity; F is the fluorescence intensity at time t; F_0 is the mean of ≥10 determinations of F during the control period. However motile, irregular loading dye and even dead sperms eliminated from the analysis.QD at 10 and 50 (µM) added to the imaging chamber by perfuse it through a multiple individual plastic tubes connecting to the perfusion system.

3.6 Estimation of Sperm Motility, Abnormality, Dead Sperms

Sperm motility estimated for control sperm having hanks solution medium (HS) and QD group that incubated with QD at 10, 50 and 500 µm from 2 hours to 14 hours respectively. The sperm abnormality and death percentage has also calculated by taking a drop of sperms of each control and treated sperms sample added separately on a glass slide, then sperms smear dried in air, stained with nigrosine dye and examined under the light microscope. The dye will penetrate the dead sperms through its cell membrane which give the sperms the color of nigrosine dye as compared with live sperms has transparent color. The percentage calculated by counted how many dead sperms have founded /100 sperms in every glass slide under microscopic observation the result is represented as percentage (%) of dead sperms. The morphological appearance and the percentage of abnormal recorded too by the same method.

3.7 Statistical Analysis

Data analysis was performed using Image-J software, raw intensity values was imported into sigma plot, analyzing program. Data represented as mean ± St. Error.

4. Results

4.1 Effect of Quinidine incubation on testicular tissues and seminiferous tubule:

The male gonads treated by QD 500µm for 14h, showed a severe bilateral atrophy, in which the basement membranes of the seminiferous tubules detached. The seminiferous tubules considerably reduced in diameter and almost entirely lined by spermatogonial cells and a few spermatocyte cells noted. Differentiated spermatozoa could not be detected as showed in Fig. (1)

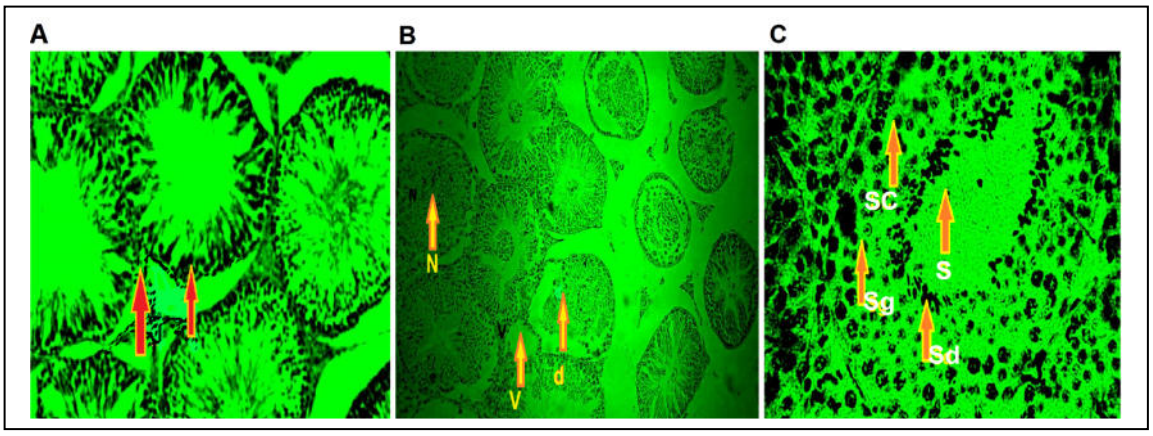

Figure 1. Section in rat testes for control and treated tisssue incubated with QD at concentration 500 µm for 14 hours. (A) untreated-control ,the orange arrows denotes a normal seminferous tubules with normal spermatocyets. While in (B ,C) QD-treated , the orange arrows denotes (Sg-spermatogonia, Sc- spermatocye, Sd-spermtid, S-sperm, N-necrosis, V-vaculation, d-degeneration

4.2 Effect of Quinidine incubation on sperm parameters:

A significant ($p<0.05$) decrease in sperm motility found in QD treated sperms 500µm for 2 hours compared to controls. In addition, the QD treated sperms showed a significant increase in sperm abnormality and elevation in sperm death percentages. Abnormal sperm shape, such curly and looped tails seen in Fig (2). Other various sperm shape morphologies, include lance-shaped, banana-shaped and balloon-shaped also observed but in a closer examination (not seen in fig.2. In addition, sperms of the control group showed a significantly lower ($p<0.05$) sperm abnormality and sperm death compared to QD treated sperms. Otherwise, it has reversible recovery after hanks (HS) medium wash containing 2mM Ca^{2+} at pH 7.4.

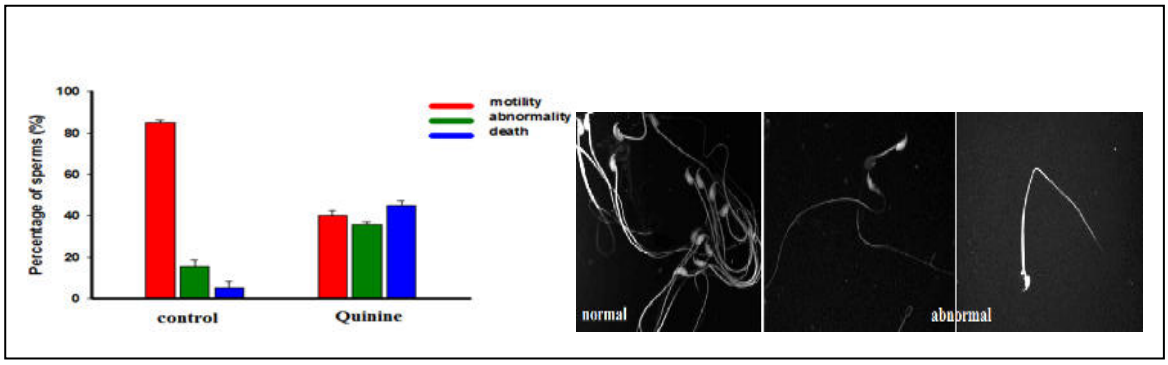

Figure 2. Illustrated the analysis of sperm samples from control and QD treated sperms incubated with QD 500µm for 2 hours.

4.3 Quinidine has Reversible Effect on Intracellular Ca^{2+} [Ca^{2+}]i in Treated Sperms:

In fig (3), vertical bars in (A) showed inhibition of [Ca2+]i entry into sperm represented by a decrease in the % of ΔF:F0 ratio of treated sperms with Quinidine 10µm 20% lower than from control conditions. After HS wash shows 88% recovery state. (B) the % of ΔF: F_0 ratio caused by Quinidine concentration 50µm 35 % followed by 75% recovery after wash the tread sperms with HS medium containing 2mM Ca^{2+} at pH 7.4.

Lower panel in (C) shows pseudo-color images (warm red colors show high [Ca2+]i of Fluo4-AM fluorescence response in a 'typical' sperm cell at various points in the experiment. The first image is prior to treatment, the second image during 10 µm QD treatment, and the third image is during washing with HS.

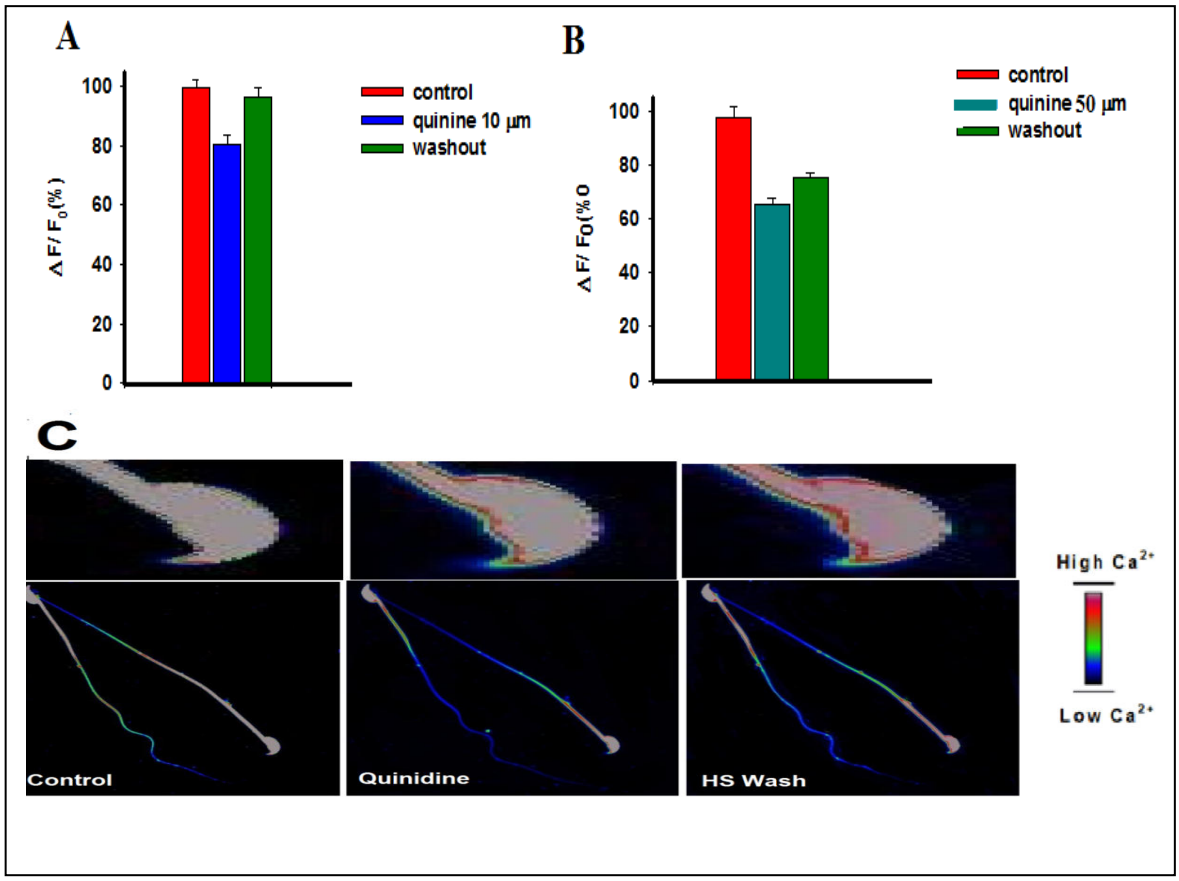

Figure 3. Ca^{2+} changes of sperm samples of control, QD treated sperms through perfuse QD 10 µm directly to sperms by perfustion system fallowed by Hanks (HS) medium wash at pH 7.4.

5. Discussion

There is a Quinine is a wide spectrum channel blocker affecting voltage-sensitive K^+ channels as well as Ca^{2+} activated one (Grinstein and Foskett 1990, Kuriyama, Kitamura et al. 1995). A study has been reported that the treatment of wild-type spermatozoa with known blockers of cell volume regulation as quinine which lead to angulated flagellum of sperm which have been observed infertile c-ros Knockout mouse (Yeung, Sonnenberg-Riethmacher et al. 1999). Moreover, quinidine has also been reported to have effects on sperm function, changing the volume regulation, decreasing velocity, and affecting mucus penetration and migration (Yeung and Cooper 2001, Barfield, Yeung et al. 2005, Barfield, Yeung et al. 2005). The sensitivity of sperm towards the effects of quinidine was still unknown. Otherwise the effective concentration of quinidine have been reported at as low as 20µM (Yeung and Cooper 2001), this is mainly within the range over which observe that quinidine, when applied extracellular, Slo3 current inhibited. The morphological changes as showed in the current study that caused degenerative changes in testicular tissue and destruction of semniferous tubules after incubation in medium containing 500 µm of QD for 14 hours. Another expectation is a mechanism of QD induced testicular damage through inhibition of protein synthesis as it's has been confirmed by Agrawal et al. (2002). To produce hydrogen bound a complex with double-stranded DNA thus inhibiting strand separation, transcription and protein synthesis (Agrawal, Tripathi et al. 2002).The present study has shown that, the mice sperms are susceptible to QD at concentrations 500 µm for 2h, sperms shape, motility even increase in the numbers of sperms mortality. Sperm has ability to maintain cellular volume by a particular mechanism known as the regulatory volume decrease (RVD). Yeung and Cooper (2001) reported that QD was active in media of both 310 and 330 mmol/kg (Yeung and Cooper 2001). This may induce the angular tail in sperms (Yeung, Anapolski et al. 2003) this can explained the abnormal tail of treated sperms with QD 500 µm after incubation for 2hours.

Our results showed Ca^{2+} decrease in sperms treated with QD at both concentration 10 and 50 µm as showed in fig.3 (A and B), with induced a completely reversible reduction in the intracellular Ca^{2+} of sperms at the lower concentration 10 µM, but a partially non complete reversible at the concentrations 50 µM. Before

applying QD, there were the stronger flurorensence intensities in control sperms. With the application of QD at 10 µm concentrations, the flurorensence intensities in wt sperms were becoming much weaker, and then got a part of recovery in intensity during washout with HS for a few minutes (Fig 3. C). Quinine examined in earlier studies on β-cells as a blocker of Ca^{2+} activated K^+ channels(Atwater, Dawson et al. 1979), also inhibit Ca^{2+} activated and ATP-regulated K^+ channels (Bokvist, Rorsman et al. 1990), the effects of quinine on the Ca^{2+} activated K^+ channel had a rapid onset and were fully reversible (Bokvist, Rorsman et al. 1990).CatSper channel complex may also mediate the effect of some other important sperm function regulators. For example, CatSper has been established to be the principal calcium channel in human spermatozoa (Strünker, Goodwin et al. 2011). Collective evidences have shown that CatSper is also essential for human male fertility. First, CatSper1-3 mRNA were recognized by reverse transcriptase polymerase chain reaction to be present in normal human sperm (Li, Liao et al. 2006). CatSper is the principal Ca^{2+}channel in human sperm, being commonly known as the most significant calcium channel necessary for mammalian male fertility (Smith, Syritsyna et al. 2013). It explains the blocking of this channel by Quinidine will lead to disturb and lower the Ca^{2+} level in sperm treated by QD. The inhibitory effect of QD on sperm metabolism (measured by production of lactic acid and CO_2) and motility have reported (Trifunac and Bernstein 1982). In another study calibrating the spermicidal activity of QD, 100% of human sperms immobilized within 20 s of incubation with the alkaloid (Garg, Doncel et al. 1994). The option for designing a male contraceptive, more things should be take place including efficacy, safety, reversibility, and ease of delivery (Nass and Strauss 2004), The vital properties of CatSper channel on sperm (for review see Navarro et al (Navarro, Kirichok et al. 2008) make this attractive proteins target for contraceptive development. Other findings concluded that QD had harmful effects on rats' seminiferous tubules and probably caused disorder in spermatogenesis and suggested. The needs are for the exploration of the place of QD as a male contraceptive agent in humans (Osinubi, Akinlua et al. 2004). As consequently, additional work is required to clarify the mechanism of QD and find out the outlook of QD as a male contraception.

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