Acute chloroquine poisoning: A comprehensive experimental toxicology assessment of the role of diazepam

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Abstract

Background and Purpose: Resurgence in the use of chloroquine as a putative treatment for COVID-19 has seen recent cases of fatal toxicity due to unintentional overdoses. Protocols for the management of poisoning recommend diazepam, although there are uncertainties in its pharmacology and efficacy in this context. The aim was to assess the effects of diazepam in experimental models of chloroquine cardiotoxicity. Experimental Approach: In vitro experiments involved cardiac tissues isolated from rats and incubated with chloroquine, alone, or in combination with diazepam. In vivo models of toxicity involved chloroquine administered intravenously to pentobarbitone-anaesthetised rats and rabbits. Randomised, controlled interventional studies in rats assessed diazepam, clonazepam and Ro5-4864 administered: (i) prior, (ii) during, and (iii) after chloroquine; and the effects of diazepam: (iv) at high dose, (v) in urethane-anaesthetised rats, and (vi) co-administered with adrenaline. Key Results: Chloroquine decreased the developed tension of left atria, prolonged the effective refractory period of atria, ventricular tissue and right papillary muscles, and caused dose-dependent impairment of haemodynamic and electrocardiographic parameters. Cardiac arrhythmias indicated impairment of atrioventricular conduction. Studies (i), (ii) and (v) showed no differences between interventions and control. Diazepam increased heart rate in study (iv) and, as with clonazepam, also prolonged the QTc interval in study (iii). Combined administration of diazepam and adrenaline in study (vi) improved cardiac contractility but caused hypokalaemia. Conclusion and Implications: Neither diazepam, nor other ligands for benzodiazepine binding sites, protect against or attenuate chloroquine cardiotoxicity. However, diazepam may augment the effects of positive inotropes in reducing chloroquine cardiotoxicity.

Keywords:

Chloroquine, diazepam, poisoning, drug overdose, antidote

What is already known

Acute chloroquine poisoning manifests as cardiotoxicity and is often managed using diazepam.

What this study adds

Diazepam does not attenuate the effects of chloroquine in isolated cardiac tissues, nor in vivo.

Clinical significance

Inotropic support, which is essential for chloroquine poisoning, may be potentiated with diazepam.

Introduction

Chloroquine and hydroxychloroquine are being repurposed for use as treatment options for coronavirus disease 2019 (COVID-19) (Ferner and Aronson, 2020). The Food and Drug Administration sanctioned their emergency use in the USA (FDA, 2020), and clinical guidelines in Belgium, China, France, India, Iran,

Italy, South Korea, and The Netherlands make recommendations for uses ranging from prophylaxis (Indian Council of Medical Research, 2020) to the treatment of the most severely affected patients.

Case reports of cardiotoxicity and fatal poisoning relating to the use of chloroquine and hydroxychloroquine for COVID-19 have emerged (Binding, 2020; Agence Régionale de Santé, 2020; Xuan, 2020; Busari and Adebayo, 2020; SimpliCity, 2020). The acute toxic effects of these drugs are well recognised (WHO, 2016), and relate to their cardiotoxic effects of widening of the QRS complex, atrioventricular block, ventricular arrhythmias, negative inotropy, hypotension and severe hypokalaemia, which occur within 1-3 hours of ingesting doses >2g in adults. Without intensive, supportive treatment, circulatory collapse and death can rapidly follow acute overdose. Mortality due to acute toxicity is high, with 134 of the 387 cases reported in the literature between 1955 and 1975 (Bondurand et al., 1980), and a further 135 from 335 suicide attempts (Weniger and World Health Organization, 1979) resulting in death.

Current recommendations for the management of acute toxicity include ensuring adequate ventilation, gastric lavage, administration of activated charcoal, adrenaline for its inotropic and vasoconstrictor effects, diazepam, and correction of metabolic acidosis and hypokalaemia (Jones, 2015). The observation in 1976 of a patient who took 5g of chloroquine together with 500mg of diazepam, and survived without symptoms of chloroquine toxicity (Djelardje, 1976), drew attention to the possible role of diazepam in chloroquine poisoning. Subsequent case reports (Jaeger et al., 1987; Rajah, 1990; Meeran and Jacobs, 1993) and a prospective non-randomised trial (Riou et al., 1988a), in which the odds of survival significantly favoured diazepam therapy, led to the recommendation of diazepam in the management of acute chloroquine toxicity. However, there remains controversy given some conflicting evidence of benefit (Damaziere et al., 1992; Clemessy et al., 1996) and limitations in study designs (Yanturali, 2004).

Experimental toxicity studies are also inconclusive. Crouzette et al., (1983) demonstrated that an intraperitoneal injection of diazepam caused a significant decrease in the mortality of rats treated with chloroquine. Riou et al., (1988b) observed an improvement in haemodynamics and a correction of the QRS interval prolongation when diazepam was administered to chloroquine-intoxicated pigs. Gnassounou et al., (1988) observed that clonazepam protected anaesthetized rats against chloroquine toxicity, and that diazepam – but not the translocator protein (TSPO) agonist Ro5-4864 (4'-chlorodiazepam) – protected against the decrease in contractions observed when guinea-pig atria were exposed to chloroquine. In other studies, however, diazepam failed to improve the mechanical performance of rat cardiac papillary muscle exposed to chloroquine (Riou et al., 1989); and was ineffective in reversing chloroquine toxicity in anaesthetized rats (Buckley et al., 1996).

It would therefore appear that the effectiveness of diazepam in reversing chloroquine toxicity is equivocal and that the mechanism(s) by which diazepam may exert its effects remain unclear. Due to the resurgence in the use of chloroquine and its structural analogue hydroxychloroquine for COVID-19, the aim of the present study was to investigate the potential cardioprotective effects of diazepam in experimental models of chloroquine toxicity.

Methods

In vitro methods

A series of experiments was conducted to assess the effects of chloroquine and diazepam, alone and in combination on the contractility, refractoriness and beating rate of isolated rat cardiac tissues.

Animals

All animal care and experimental procedures was performed in accordance with the UK Animals (Scientific Procedures) Act 1986, approved by the institutional ethical review committee, and conducted under the authority of project licences held at the University of Liverpool. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny et al., 2010; McGrath and Lilley, 2015).

Male Wistar rats were bred in the departmental animal unit (the Nuffield Joint Facilities) or, in exceptional circumstances of supply shortage, acquired from the Biomedical Services Unit, Faculty of Medicine or the

Department of Veterinary Pathology. Rats were kept under conditions of 12 hour light / dark cycle at 20degC with food (CRM diets, SDS, Witham, Essex) and water available *ad libitum* . The optimal weight range for experimental use was 200-400 g.

Tissue preparation

Rats were administered 1000 IU kg^{-1} of sodium heparin by an intraperitoneal injection. After 15 minutes, they were stunned by a blow to the head, exsanguinated, and hearts were excised. Isolated atria, ventricular strips ([?]2mm in width) dissected longitudinally towards the apex of the heart, and right papillary muscles were prepared and suspended in 30 ml organ baths, containing (in mM), NaCl 119; KCI 3.8; $MgS0_4$ 1.18; $KH_2PO_4$1.18; $NaHCO_3$ 25; $CaCl_2$ 1.9 and D-Glucose 10.0, gassed with 95% O_2, 5% CO_2 (BOC medical gases, Guildford), and maintained at 37degC. Each preparation was subjected to a resting diastolic tension of 10 mN and stimulated with square wave pulses of 5 ms duration at a frequency of 1 Hz via a Grass S48 or S88 stimulator (Quincy, Massachusetts). Tissues were stimulated at twice threshold voltage [?]15 V. Right atria were allowed to equilibrate such that spontaneous, rhythmic beating occurred. In all cases, tissues were washed periodically throughout the stabilisation period.

Measurement of cardiac parameters

Contractions were measured isometrically via Dynamometer UF1 transducers (sensitivity range, 559 mN) connected to Lectromed 5230 preamplifiers (Letchworth, Hertfordshire). The beating rate of right atria was measured with a Lectromed 5250 ratemeter preamplifier. These were housed within a MT8P preamplifier unit which relayed signals to a MT6 thermal-pen-recorder giving an output on heat sensitive paper. Each channel was calibrated such that a full scale deflection of 20 mN could be observed. Time to peak tension was measured from the onset of electrical stimulation to the peak of the contraction (Penefsky, 1994). The effective refractory periods (ERP) of left atria, right papillary muscles, and ventricular strips were measured using a modification of the extra stimuli method (Reuter and Heeg, 1971).

Experimental protocol

A target of 6 samples of each cardiac tissue were assigned at random to one of four concentrations of chloroquine base (1, 10, 30 or 100 μM, dissolved in Krebs solution) in the presence of the vehicle for diazepam (1% v/v propylene glycol). Spontaneously beating right atria were exposed only to 30 μM chloroquine. In a separate experiment, fresh tissues (target of 6 per group) were incubated with diazepam at concentrations of 1, 10 or 100 μM for 30 minutes before the addition of 30 μM chloroquine.

In vivo methods

Experimental models of toxicity were developed in spontaneously breathing rats, ventilated rats and ventilated rabbits, in which chloroquine was infused at different rates, and measurements taken of haemodynamic and electrocardiographic parameters. Interventional studies were then conducted in which chloroquine-intoxicated rats were treated with combinations of diazepam, clonazepam, Ro5-4864, adrenaline or vehicle control.

Experimental protocol

In developing a model of experimental toxicity, animals (6 per group) were allocated at random to different doses of chloroquine diphosphate dissolved in 0.9% w/v NaCl. Non-ventilated rats were randomised to intravenously infused doses (calculated as chloroquine base) of 0.5, 1, 2 or 4 mg kg^{-1} min^{-1}; ventilated rats 1, 2, or 4 mg kg^{-1} min^{-1}; and rabbits 0.5, 1 or 2 mg kg^{-1} $min^{-1}$for a maximum period of 60 minutes or until death, after an initial period of stabilisation of at least 20 minutes.

Six interventional randomised controlled trials were subsequently conducted to assess the efficacy of diazepam, clonazepam and Ro5-4864: (i) prior, (ii) during and (iii) after chloroquine intoxication (Table 1); and the effects of diazepam: (iv) in high dose, (v) in non-barbiturate anaesthetised rats, and (vi) co-administered with adrenaline. Six rats were randomised to each intervention group within each of these studies. Benzodiazepines (and vehicles) were administered as a slow intravenous bolus over 2 minutes.

Drug doses

Diazepam was administered in a 2 mg kg^{-1} intravenous bolus dose, based on Riou et al., (1988b) and dose recommendations for human cases of overdose (Jones, 2015); and 10 mg kg^{-1}in the study of high dose diazepam. The doses of clonazepam (1.1 mg kg^{-1}) and Ro5-4864 (0.16 mg kg^{-1}) were chosen to have the equivalent GABAergic and non-GABAergic activity, respectively, to 2 mg kg^{-1} of diazepam (Wang et al., 1984). Adrenaline was administered at 0.3 µg $kg^{-1}min^{-1}$, consistent with the recommended dose for the clinical treatment of overdose (Jones, 2015), and which produces a 50% increase in maximum rate of left ventricular pressure (LV $+dP/dt_{max}$) in anaesthetised rats (Latini et al., 1988).

Animals

Male Wistar rats (as above) and female New Zealand White rabbits, which were either bred in the departmental animal unit or purchased from Harlan Interfauna (Huntingdon, Cambridgeshire), were used. Rabbits were housed under ambient conditions of a 12 hour light/ dark cycle at 18°C with food (R14 from SDS, Witham, Essex) and given amprolium HCI 7.68 % w/v and ethopabate 0.49% w/v (1.5 ml per 500 ml) drinking water for 5 days as a prophylaxis against *coccidiosis* infection. For rabbits, the optimal weight range for experimental use was 2-3 kg.

Anaesthesia

Anaesthesia was induced in rats with sodium pentobarbitone 60 mg kg^{-1} intraperitoneally and, once venous access was established, maintained with intravenous boluses of 3 mg, as required. In intervention study (v), urethane was prepared as a 15% w/v solution in isotonic saline, and administered as an intraperitoneal dose of 1.4 g kg^{-1}.

Neuroleptanalgesia was induced in rabbits by an intramuscular injection of 0.5 ml kg^{-1} Hypnorm (0.315 mg ml^{-1} fentanyl citrate and 10 mg ml^{-1}fluanisone). Surgical anaesthesia was achieved by administering sequential 4 mg boluses of sodium pentobarbitone into the marginal ear vein, then 12 mg boluses via a cannulated femoral vein, as required, upon commencement of ventilation.

Surgical preparation

Femoral veins were cannulated for venous access for drug administration. The right common carotid and a femoral artery were accessed for measurement of left ventricular pressure and recording of blood pressure using a Druck PDCR 75 or a Bell and Howell type 4-422-0001 pressure transducer. A tracheotomy was performed to facilitate respiration, and a wide bore cannula secured in place. Subcutaneous stainless steel needle electrodes were inserted to each limb for the recording of the electrocardiogram (ECG). All animals were maintained at a rectal temperature of 37 °C.

Mechanical ventilation

Ventilation necessitated a thoracotomy at the fifth intercostal space as, in closed-chest rats, excessive contractions of the diaphragm and intercostal muscles was found to prevent effective respiration. A positive end-expiratory pressure was exerted and air ventilation provided at 54 strokes min^{-1} (3-4.5 ml per stroke) using a Harvard Bioscience small animal respirator. Rabbits were ventilated with air at 38 strokes min^{-1} (13-18 ml per stroke). A thoracotomy was not necessary in anaesthetized rabbits. Blood gases were measured using a Corning 158 or 850 pH / blood gas analyser. Stroke volumes were adjusted for pre-drug PO_2>80 mmHg and PCO_2 >30 mmHg.

Exclusion criteria

Animals were excluded with pre-drug mean arterial blood pressure <60 mmHg (in anaesthetized rats) or <40 mmHg (in ventilated rabbits); arterial PO_2 of <70 mmHg; arterial PCO_2 <25 or >40 mmHg; or if arrhythmias occurred during the stabilisation phase of the experiment. In the randomised trials, rats were excluded if they died prior to the administration of chloroquine in trial (i), or the intervention in trials (ii) to (vi).

Measurement of cardiovascular parameters

Arterial blood pressure, left ventricular pressure, and its first derivative (LV $\pm dP/dt_{max}$) and contractility index LV $+dP/dt_{max}$ / P, left ventricular end-diastolic pressure, heart rate and ECG (lead II) were measured and recorded using Lectromed systems (Letchworth, Hertfordshire) or a Grass 79D recorder (Quincy, Massachusetts) connected to a Po-Ne-Mah digital data acquisition system (Linton, Diss, Norfolk) and recorded at a sampling rate of 1000 Hz.

Whole blood concentration of chloroquine

Blood samples for the determination of chloroquine concentration were obtained from trial (iv). Approximately 250 µl arterial blood samples were drawn after 25, 45 and 60 minutes of chloroquine infusion from 6 rats for the analysis of whole blood chloroquine concentrations. Four 50 µl aliquots were accurately pipetted on to a sheet of Whatman grade 3 blotting paper, and protected from light exposure.

Standards were prepared by adding aliquots of chloroquine, giving final concentrations ranging from 0 to 30 µM, on to chloroquine-free blood spots. All samples were carefully cut from the surrounding paper, macerated and placed in individual glass vials containing 50 µl of a 1 µg ml^{-1} solution of 7-chloro-4-(5-diethylamino-1-methylpentyl-amino)-quinoline diphosphate to serve as an internal standard, and 3 ml of 0.2 M HCI. The vial contents were vortexed, allowed to settle, filtered and added to 0.5 ml of 5 M NaOH and 2.5 ml each of methyl-tertiary-butyl ether (MTBE) and hexane. This was centrifuged and the organic layer separated. Fresh MTBE and hexane were added to the remaining aqueous phase, and the extraction procedure repeated. The solvent was evaporated from each organic sample by heating combined with a gentle flow of dry nitrogen. Samples were then stored at -20°C.

Chloroquine was detected using an Isochrom LC Spectra-Physics pump equipped with a Rheodyne injector, a Spectra 100 fluorescence detector and a Chromjet integrator. The excitation wavelength was 340 nm and a 370 nm emission filter was used (Looareesuwan et al., 1986). The column (0.25m x 4.6 mm internal diameter) was packed with Spherisorb silica (5 µM particles; Capital HPLC) and eluted with an isocratic mobile phase consisting of acetonitrile: methanol: diethylamine (94: 5.5: 0.5), flowing at 1.5 ml min^{-1}. The limit of detection for chloroquine was 3.1 nM and the precision of the method was 3.5% at 156 nM.

Biochemical measurements

In randomised trial (vi), arterial blood samples were analysed for blood gases, pH and electrolyte (K^+, $Na^+$and free Ca^{2+}) concentrations using a Corning 850 analyser. Blood samples of approximately 200 µl were collected at baseline, pre-intervention and 30 minutes post-intervention.

Analysis of electrocardiographic parameters

ECG interval measurements were made manually, and based on the average of 4 successive ECG complexes for each recording time point. The QRS interval was measured from the onset of the Q wave (or R wave if no Q was visible) to the point at which the ST segment bisected the isoelectric line. The QT interval was corrected (QTc) using Bazett's formula (Bazett, 1920).

Drugs and reagents

All salts for Krebs solutions were of AnalaR grade or higher and obtained from BDH, Poole or Fisons, Loughborough. Chloroquine diphosphate, (±) adrenaline HCl, ethyl carbamate (urethane), Tween 80, N,N-dimethylacetamide and propylene glycol were obtained from Sigma, Poole. Heparin sodium (Multiparin 5000 IU ml^{-1}) injection was obtained from CP Pharmaceuticals, Wrexham. MTBE was purchased from Fisons, Loughborough; Ro5-4864 was purchased from Fluka Chemika, Gillingham, Dorset; Hypnorm (fentanyl / fluanisone) was from Janssen Animal Health, Petteridge, Kent; Amprol Plus (Amprolium HCl / ethopabate) was from Merk Sharp & Dohme, Hertfordshire; sodium pentobarbitone from RMB Animal Health Ltd, Dagenham. 7-chloro-4-(5-diethylamino-1-methylpentyl-amino)-quinoline diphosphate was a gift from the Walter Reed Army Institute of Research, Washington D.C.; and clonazepam and diazepam were gifts from Hoffman-La Roche, Basel.

Data and statistical analysis

Data are presented as means ± standard error of mean (SEM) of the average of observations over the time course of the experiment. Data were tested for normality using Shapiro-Wilk tests. For multiple ([?]3) comparisons, data were compared by one-way analysis of variance followed by a Bonferroni modified t -test, or by Kruskal-Wallis tests if non-normally distributed. Comparisons of two groups of data used either an unpaired t -test for normally distributed data, or a Mann-Whitney U test for skewed data or where there was a significant difference between the variances of each group. Differences between groups in the time to onset of arrhythmias were analysed by log-rank tests. A p-value less than 0.05 was considered statistically significant. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018). Statistical analyses were performed using Arcus Pro-Stat version 3.12.

Results

In vitro cardiac tissues

Tissues from 57 rats (272 +- 4 g) were used. Decreases in the developed tension of left atria were observed with chloroquine at the highest concentration of 100 μM. The negative inotropic effect was time-dependent, with maximal changes observed by 30 minutes. Chloroquine did not significantly alter the developed tension or time to peak tension of right ventricular strips or papillary muscles; but increased the time to peak tension in atria (70 ± 4 ms with chloroquine (100 μM) compared to 54 ± 2 ms in the control group; $p<0.05$). Chloroquine prolonged the ERP of all tissues. In left atria, for instance, the pre-chloroquine ERP was 41 ± 2 ms (in the 30 μM group), which increased to 72 ± 9 ms after 30 minutes exposure to chloroquine ($p<0.001$).

Diazepam alone was without effect on papillary muscles or ventricular tissue other than a small but significant increase from 65 ± 2 to 74 ± 2 ms in the time to peak tension of contracting right ventricular strips at 100 μM. However, diazepam 100 μM evoked a positive inotropic response and prolonged the ERP of left atria, and had a negative chronotropic effect on right atria (213 ± 11 versus 290 ± 14 beats min^{-1}; $p<0.001$).

Diazepam in the concentration range of 1 to 100 μM did not appear to protect against the effects of 30 μM chloroquine (Table 2). At the highest concentration, diazepam lengthened the ERP and extended the time to peak tension in left atria, and reduced rate of beating right atria.

In vivo models of experimental toxicity

Haemodynamic effects

In both rats and rabbits, marked, dose-dependent decreases in systolic and diastolic blood pressures were observed during continuous infusions of chloroquine (Figure 1). In spontaneously breathing rats, the highest dose of chloroquine (4 mg $kg^{-1}min^{-1}$) caused the most pronounced effect, with a reduction in systolic pressure from 123 ± 15 to 37 ± 6 mmHg occurring during the first 8 minutes. Similar reductions in pressure were observed in ventilated rats receiving chloroquine although baseline values were lower, as expected in thoracotomised rats. Equivalent depressor responses in rabbits occurred with approximately twofold less chloroquine.

Chloroquine caused dose-dependent negative inotropy in both species. Reductions in LV $+dP/dt_{max}$ during the first 2 to 4 minutes of infusion seemed more pronounced than reductions in blood pressure. For example, a 47% reduction in LV $+dP/dt_{max}$occurred during the first 2 minutes of infusion at 2 mg kg^{-1} min^{-1} compared with a 15% reduction in diastolic pressure for the same period in non-ventilated rats. Cardiac lusitropy (LV $-dP/dt_{max}$) declined in a parallel manner to the negative inotropic response. Increases in left ventricular end diastolic pressure were observed with chloroquine in non-ventilated rats and ventilated rabbits.

Chloroquine caused a similar dose-dependent bradycardia over the time course of the experiment in both ventilated and non-ventilated rats for the corresponding doses. In rabbits, however, heart rate declined abruptly by approximately half at time points corresponding to the onset of arrhythmias.

Electrocardiographic effects

In rats, increases in the PR intervals occurred with all doses of chloroquine, and in proportion to the cumulative dose received. For example, the PR interval increased from 52 ± 3 to 68 ± 4 ms during the first 12 minutes in ventilated rats receiving 1 mg kg^{-1} min^{-1} chloroquine, and from 50 ± 2 to 68 ± 4 ms during the first 6 minutes at twice the infusion rate, with both groups receiving a total of 12 mg kg^{-1} of chloroquine over these periods. Chloroquine also caused a dose-dependent increase in the PR interval in rabbits.

Chloroquine broadened the QRS complex in all animals (Figure 2), although this was not as pronounced with the slower infusion rates as the changes in PR duration. In ventilated rats, for example, the QRS duration increased by 17% in the first 30 minutes of chloroquine being infused at 1 mg kg^{-1} min^{-1} while a 30% increase in PR interval occurred over the same period.

QT interval prolongations were observed with high infusion rates, but these were not as apparent when the QT was corrected for rate effects. A substantial increase in QTc occurred only with 2 mg kg^{-1} min^{-1} in ventilated rats.

Chloroquine induced arrhythmias in 34/38 rats. Typically, impairment of atrioventricular (AV) conduction leading to varying degrees of AV block, was associated with ventricular ectopy. Ventricular bigeminy sometimes preceded episodes of ventricular tachycardia in the latter stages of infusion. Ventricular tachycardia was commonly triggered by 'R on T' depolarizations. In three rats, the ventricular tachycardia was polymorphic with characteristic features of torsade de pointes. Two rats in each of the ventilated and non-ventilated groups did not experience cardiac arrhythmias at the lower dose of 0.5 mg $kg^{-1}min^{-1}$.

In all 16 rabbits, arrhythmias presented as Mobitz type II, second degree AV block with a conduction ratio of 2:1 (two P waves for each QRS complex). The onset of arrhythmias was dose dependent, and with higher degrees of block eventually occurring at the faster infusion rates. These largely degenerated to ventricular tachycardia and fibrillation.

In vivo interventional trials

(i) Efficacy of ligands for benzodiazepine binding sites (before infusion of chloroquine)

Twenty-seven rats entered in the study, but 3 died immediately following the administration of clonazepam and were excluded. There were no differences between pre- and 10 minutes post-drug haemodynamics or ECG parameters or between randomised groups with the administration of diazepam, clonazepam, Ro5-4864 or vehicle. In the presence of these agents, chloroquine reduced blood pressure, heart rate and contractility index; and increased the PR, QRS and QTc intervals (Table 3). There were no significant differences in these parameters, or in the time to developing cardiac arrhythmias: 18.1 ± 2.8, 15.8 ± 1.6, 17.5 ± 1.2, 17.8 ± 2.3 minutes, between the control, diazepam, clonazepam and Ro5-4864 groups, respectively.

(ii) Efficacy of ligands for benzodiazepine binding sites (during infusion of chloroquine)

Twenty-five rats entered the study, but one was excluded having died before being administered the intervention. Following 30 minutes of chloroquine infusion there were similar, significant changes in haemodynamics and ECG intervals across intervention groups (Table 3). There were no differences in any of the measured parameters between intervention and groups following the administration of treatment; however all rats survived without developing arrhythmias.

(iii) Efficacy of ligands for benzodiazepine binding sites (after infusion of chloroquine)

Three rats were excluded as they died prior to the end of chloroquine infusion. A further 4 rats died before the end of the experiment but were included in the study: 1 each from the clonazepam and control groups, and 2 from the Ro5-4864 group. There was an imbalance in pre-chloroquine baseline QRS intervals, with higher values in those randomised to Ro5-4864 compared with other groups. Over 15 minutes, chloroquine caused significant changes in all cardiovascular parameters in all randomised groups; however, there was a difference between groups in the QTc interval, which was shorter in the control versus diazepam, clonazepam

or Ro5-4864 groups. Following the cessation of chloroquine administration, and administration of randomised treatment, all parameters rapidly returned to baseline values in all groups (Table 3). Heart rate was higher, and QTc interval prolonged in both the diazepam and clonazepam groups, compared with control.

(iv) Efficacy of high dose diazepam (during infusion of chloroquine)

In contrast to trial (ii), an infusion of chloroquine for 30 minutes did not cause significant changes in all haemodynamic or ECG parameters. Chloroquine did not reduce mean BP, heart rate or increase the QTc interval significantly in those randomised to diazepam; and did not increase the QRS interval in either group. Following intervention, heart rate was significantly increased in the diazepam group; other parameters were no different between treatment groups (Table 3).

The whole blood chloroquine concentration in these rats was 12.2 ± 0.8 μM after 25 minutes of infusion (1 mg $kg^{-1}min^{-1}$), 14.3 ± 0.9 μM after 45 minutes, and 16.3 ± 1.1 μM after 60 minutes.

(v) Efficacy of diazepam (during infusion of chloroquine) with a non-barbiturate anaesthetic

Over 30 minutes of administration, chloroquine only significantly affected the PR and QRS intervals. There were no subsequent differences between groups, following administration of diazepam or vehicle control, in any of the measured parameters (Table 3). A further randomised trial was initiated with chloroquine infused at a higher rate of 2 mg kg^{-1} min^{-1} in order to evaluate the effects of diazepam with more pronounced toxicity, however, 5 of the first 9 rats recruited died, and the study was terminated.

(vi) Efficacy of diazepam and adrenaline (during infusion of chloroquine)

Twenty-seven rats was included, but 3 died before the end of the experiment – one from each of the control, diazepam, and diazepam + adrenaline groups. During the first 30 minutes of infusion, chloroquine caused significant changes in all parameters, with the exception of the QRS and QTc intervals in the adrenaline group. The lack of an effect with diazepam (2 mg kg^{-1}) alone was consistent with trial (ii). The effects of adrenaline alone did not deviate significantly from the control group in any parameter other than the QRS interval, but this was not prolonged following chloroquine (Figure 2). The combined administration of diazepam and adrenaline resulted in an improvement of cardiac contractility compared to the control and diazepam groups but not the adrenaline group (84 ± 3 versus 78 ± 8 s^{-1}; p=0.063). No significant differences were observed in the other parameters (Table 3).

Pre-chloroquine potassium concentrations were in the range expected for rats (Burns and De Lannoy, 1966). Chloroquine alone did not cause any significant changes in arterial PO_2, PCO_2 or pH values over a period of 30 minutes infusion. The combined administration of diazepam and adrenaline, however, reduced PO_2 when compared to pre-intervention values but not when compared to the other groups. Chloroquine did not alter electrolyte concentrations; but intervention groups containing adrenaline were more hypokalaemic than the diazepam and control groups (Table 4).

Discussion

The findings from the *in vitro* studies are congruent with previous experiments demonstrating the acute cardiotoxic effects of chloroquine (Essien and Ette, 1986; Tona et al., 1990). At the concentrations of chloroquine used, left atria were more sensitive to detrimental effects on mechanical performance than either ventricular or papillary tissue preparations. Decreases in developed isometric tension, together with increases in times to peak tension were observed, which are indicative of impaired atrial contractility. An increase in the time to peak tension by chloroquine reflects a prolongation of one or more phases of the cardiac excitation-contraction cycle, and is consistent with the ability of chloroquine to block cardiac ion channels (Essien and Ette, 1986; Tona et al., 1990; Sánchez-Chapula et al., 2001; Rodríguez-Menchaca et al., 2008). Ikhinmwin et al., (1981) demonstrated a negative inotropic response which was reversed in the presence of increased extracellular calcium aimed to promote calcium influx via unblocked L-type calcium channels. Tona et al., (1990) demonstrated chloroquine to inhibit the Treppe response in atrial guinea pig preparations, but without effect on post-extrasystolic potentiation of contractile force, suggesting that chloroquine interferes

with cellular calcium influx upon which the Treppe response is dependent, but not the latter response which is dependent on intracellular calcium mobilisation. Increases in the refractoriness of cardiac tissues are indicative of potassium and /or sodium ion channel blockade. Using voltage-clamped cat ventricular myocytes Sánchez-Chapula et al., (2001) observed that chloroquine blocked several inward and outward membrane currents. The order of potency (1-10 μM range) was inward rectifying potassium current > rapid delayed rectifying potassium current > sodium current > L-type calcium current. Neither the transient outward potassium current nor the slow delayed rectifying potassium current were modified by chloroquine. Salinas and Cebada, (1993) also demonstrated that chloroquine blocks the inward rectifying potassium current in dog cardiac myocytes, but had no effects on either the transient outward, or the delayed rectifier currents. Other quinolone antimalarials also have known actions in modulating cardiac electrical activity, including blockade of human ether-a-go-go related gene (hERG) potassium and L-type calcium channels (Coker at al., 2000; Michel et al., 2002; Kim et al., 2010).

Diazepam had little effect on the function of myocardial tissue, other than at 100 μM, where it increased the effective refractory period and peak developed tension in left atrial preparations; and increased the times to peak tension in right ventricular strips. Diazepam inhibits phosphodiesterase (PDE) 4 (Collado et al., 1998), suggesting a possible mechanism for cardioprotection, although this occurs at lower concentrations (IC_{50} of 8.7 μM) than required to elicit responses in the present investigation. The responses of cardiac tissues to chloroquine in the presence of diazepam were no different from vehicle controls, supporting previous observations that diazepam does not attenuate the cardiac effects of chloroquine via a direct action upon the heart (Riou et al., 1989).

The *in vivo* experimental models of chloroquine toxicity indicated that rabbits and rats responded in a similar manner to chloroquine administration, with impaired cardiac contractility being the primary event in the sequalae of toxicity. Hypotension, bradycardia, changes in ECG intervals, arrhythmias and death followed, in a similar manner as described previously (Sofola, 1980). The provision of mechanical ventilation did not appear to influence the onset or the severity of these effects. Significant changes in cardiovascular function occurred in the absence of changes in either arterial blood gas levels or pH, suggesting that toxic manifestations due to chloroquine are not secondary to hypoxia. Chloroquine was about twice as potent in its toxic effects in rabbits than in rats, where whole blood concentrations were within the 10-20 μM range, and comparable with the concentrations used in the *in vitro* experiments.

The series of randomised controlled trials were designed to assess whether modulation of the $GABA_A$ receptor or other effects of diazepam might account for previous reports of reduced toxicity with chloroquine. However, diazepam, whether administered prior, during or after the administration of chloroquine or at high dose, failed to attenuate chloroquine-induced cardiotoxicity in anaesthetized rats. These results are consistent with previous studies in spontaneously breathing rats anaesthetised with thiobutobarbitone (Buckley et al., 1996), but contrast with experiments performed in conscious rats (Crouzette et al., 1983) and pentobarbitone-anesthetized, mechanically-ventilated pigs (Riou et al., 1988b). Possible explanations for these discrepancies might include the choice of species, doses of chloroquine and diazepam, and anaesthesia. Effects were similar, however, in the trial in which urethane was chosen as an anaesthetic for its lack of interaction with $GABA_A$ receptors.

Experiments aimed to differentiate any GABA mediated versus other effects of diazepam, used Ro5-4864, which has activity at the mitochondrial TSPO benzodiazepine binding site distinct from the $GABA_A$ receptors in the central nervous system; and clonazepam, which rapidly crosses the blood-brain barrier and is a potent, positive allosteric modulator of $GABA_A$receptors, while having low affinity towards TSPO. Mitochondrial TSPO is ubiquitously expressed in various tissues, including the heart with a putative role in regulating heart rate and contractility (Surinkaew et al., 2011). As neither diazepam nor either of these agents protected against or attenuated chloroquine toxicity, it is unlikely that any cardiovascular effects – in the context of chloroquine toxicity – can be attributed to interaction with benzodiazepine binding sites.

In view of the fact that the principal adverse effect of chloroquine is negative inotropy (Sofola, 1980), and the absence of positive inotropic effects of diazepam under basal conditions (and negative inotropy under

certain conditions (Zeegers et al., 1998)), the use of a positive inotrope seems essential for the improvement in the cardiac function following chloroquine toxicity. While neither diazepam nor adrenaline alone reversed any chloroquine-induced cardiovascular changes, the improvement in cardiac contractility observed with their combined administration may indicate a beneficial interaction. Studies in rat ventricular tissues demonstrated diazepam (10 μM) to augment contractility due to isoprenaline (Martinex et al., 1995), noradrenaline (Juan-Fita et al., 2003) and dopamine (Juan-Fita et al., 2006). These effects were not mimicked by GABA nor antagonized by the selective TSPO inhibitor PK11195, or flumazenil, an antagonist of the $GABA_A$ benzodiazepine binding site. Rather, they were attributed to diazepam's ability to inhibit PDE-4, the main isoenzyme responsible for the inotropic effect of β-adrenoceptor agonists in the rat myocardium. This offers a plausible mechanism for the observed effects in chloroquine intoxicated rats. However, there are differences between species in the expression of PDE-4, with a fivefold higher amount of non-PDE4 activity in human hearts compared to rodents, and this will impact on the effect of enzyme inhibition (Richter et al., 2011).

Conclusions

Although the results of this study do not offer compelling support for the use of diazepam in reducing chloroquine cardiotoxicity, evidence that it might enhance cardiac contractility when co-administered with adrenaline is plausibly explained through its PDE-4 inhibitory effects. However, adrenaline lowered whole blood potassium concentrations, consistent with agonism of β_2-adrenoceptors in skeletal muscle. As this may exacerbate chloroquine-induced hypokalaemia (Clemessy et al., 1995) and increase arrhythmogenicity, it is proposed that treatment of acute chloroquine toxicity should instead include the administration of a positive inotrope with a greater selectivity for β_1-adrenoceptors, such as dobutamine. Given that chloroquine poisoning often causes convulsions, which can be intractable, there may be additional, non-cardiovascular roles for diazepam treatment.

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