UNIVERSITY OF GHANA MEDICAL SCHOOL
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IN VIVO ASSESSMENT OF THE EMBRYOTOXICITY OF CHLOROQUINE IN THE RAT

BY

SAMUEL NII - AWULEY LARTEY

A THESIS PRESENTED TO THE UNIVERSITY OF GHANA IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF MPHIL. DEGREE IN ANATOMY.

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DECLARATION

I declare that this thesis entitled *IN VIVO* ASSESSMENT OF THE EMBRYO-TOXICITY OF CHLOROQUINE IN THE RAT submitted for the Master of Philosophy (M. Phil.) degree in Anatomy is based on work conducted by the author. This was done in the Department of Anatomy, University of Ghana Medical School of the College of Health Sciences, University of Ghana, Legon.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references. None of this work has been submitted for another degree in this or any other university.

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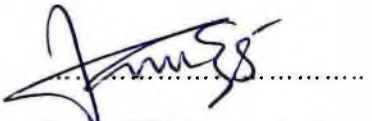
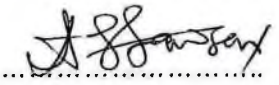


Samuel Nii-Awuley Lartey

(Student)

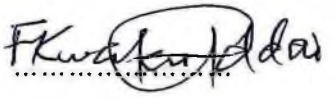
Prof. C. N.B. Tagoe.

(Supervisor)



Prof. A. L. Lawson

(Supervisor)



Prof. F.K. Addai

(Supervisor)

July 2004

DEDICATION

I dedicate this work to:

1. The Glory of God Almighty.
2. My Mom, Felicia Narkuor Kudjey For Her Sacrifice.
3. My Wife, Emefa

   and

4. Our Priceless Gifts:

   Naa-Lamley

   Nii -Awuley (Jnr.)

   Paa -Eworkoh

   Naa -Tsuishito

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ABSTRACT

The embryotoxicity potentials of normal dosage regimens of chloroquine used for therapeutic purposes were assessed in this research. Accordingly, five dosage regimens (7.5, 10.0 12.5 15.0 and 20.0 mg/kg body weight) of chloroquine diphosphate were screened by the High Performance Liquid Chromatography (HPLC) method to select those, which will provide whole blood levels in the (Sprague-Dawley) rats comparable to those achievable during therapeutic use. The dosages of 7.5, 10.0 and 12.5 mg/kg body weight produced peak levels of 1,811.80 ng/ml (3.5 μM) 1,688.31 ng/ml (3.3 μM) and 1,125.54 ng/ml (2.5 μM) respectively and were selected for the embryotoxicity studies. These were injected intraperitoneally into pregnant Sprague-Dawley rats on day $9^1/_2$ of conception to assess their possible embryotoxicities over a 48-hour period. The results suggest a possible embryotoxicity of the dosages applied, even at those relatively low concentrations. They also indicated that there was a relationship between the dosage of chloroquine injected intraperitoneally and the peak blood concentration but not the time the peak concentrations of the drug was achieved. The drug produced varying degrees of growth retardation and dysmorphogenesis in the embryos and these were generally found to be dose related. Dysmorphogenesis was assessed as 10% in the control, 16% in the 7.5 mgCQ/kg body weight, 14% in the 10.0 mgCQ/kg body weight and 17% in the 12.5 mgCQ/kg body weight treatment. The most common morphological abnormalities were

abnormal axial rotation, unfused and underdeveloped cranial neural tube, microophthalmia and abnormal otic and optic primordia. At treatments of 10.0 mgCQ/kg and 12.5 mgCQ/kg body weight there were significant reductions in yolk sac diameter to 80% and 71%, respectively. Total protein contents were also reduced to 53% and 45%, head lengths to 67% and 65%, and crown-rump lengths to 80% and 70%, of control values. There were no significant reductions however, in the number of somites and the total morphological scores. Finally, the results from the study showed that the racemic chloroquine applied was embryotoxic even at those relatively low dosages.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 BACKGROUND

Although chloroquine (CQ) was developed primarily as an anti-malarial agent, it possesses other pharmacological properties, some of which are very useful and are therefore effectively being harnessed for the welfare of mankind in general.

Its anti-inflammatory properties are well documented (Felson *et al.*, 1992). Its potency in the treatment of rheumatoid arthritis and discoid lupus erythematosus is well established (Ropes *et al.*, 1959). It is found to be very effective in combination with several intestinal amoebicides, like emetine, for the treatment of amoebic abscess and other related indications. The drug is also very useful in the suppression of some aspects of photoallergic reactions such as polymorphous light eruption and porphyria cutanea tarda, which are unresponsive to other therapies (Brogden and Heel, 1978).

1.2 THE DRUG CHLOROQUINE (CQ)

The drug chloroquine (CQ) belongs to a group of chemicals called the 4-aminoquinolines by virtue of their basic structure (Warhurst, 1987). It was found to have been synthesised and studied by the Germans in the 1930's

(Goldberg *et al.*, 1997). However, its current name only gained worldwide prominence and recognition in the treatment of malaria after the drug had proved very effective in the treatment of both avian and human-induced malarias (Chou *et al.*, 1980).

1.2.1 CHEMICAL AND BIOLOGICAL PROPERTIES

1.2.1.1 PHYSICOCHEMICAL CHARACTERISTICS

The drug may commonly be presented as chloroquine base, chloroquine hydrochloride and chloroquine phosphate. These have molecular weights of 319.88 g, 392.80 g and 515.87 g respectively.

1.2.1.2 CHEMICAL STRUCTURE

Chloroquine has a quinoline nucleus with a side chain (Figure 1) and lacks a methoxy moiety. The chlorine atom attached to position 7 of the quinoline ring confers on the drug its greatest antimalarial activity in both avian and human malarias (O`Neill *et al.*, 1998; Raynes, 1998).

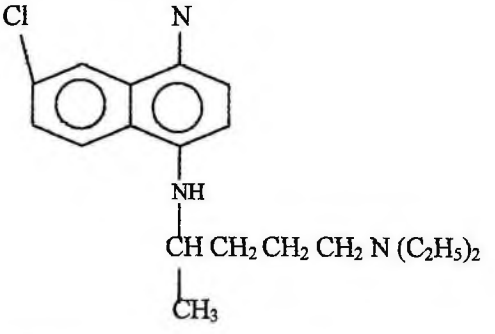


FIGURE 1: The chemical structure of the chloroquine molecule.

1.2.1.3 COMPOSITION

The drug, as being commercially presented, is a racemic mixture of two enantiomers, the (+) –CQ and (-)-CQ forms.

1.2.1.4 BIOLOGICAL PROPERTIES

These enantiomers exhibit different biological properties. For instance, the (+)-CQ enantiomer is found to exhibit both greater binding to plasma proteins and metabolic clearance than the (-)-CQ enantiomer in humans (Krishna and White, 1996).

1.3 PHARMACOKINETICS

The enantiomers have also been shown to have different pharmacokinetic properties (Ofori-Adjei *et al.*, 1986; Ofori-Adjei *et al.*, 1986; Angustijns and Verbeke, 1993) and therefore may exhibit different therapeutic effects (Haberkorn *et al.*, 1979; Fu *et al.*, 1986). Fu *et al.* (1986) further indicated that in spite of these differences, the (+) -CQ, (-) -CQ and the racemic forms are all potent in the treatment of malaria.

Chloroquine may be administered both orally and parenterally depending on the circumstances. When given orally, it is slowly and almost

completely absorbed from the gastrointestinal tract (Gustafson *et al.*, 1983). It is, however, absorbed rapidly from intra-muscular and subcutaneous sites. Chloroquine has a bioavailability of approximately 89%. It accumulates extensively within blood cells and reaches a maximum plasma concentration within three hours after its administration (Bergqvist and Domeiji–Nyberg, 1983).

Given orally once a week, either orally or parenterally, it has a peak level of about 200 ng/L (0.39 μM) and a trough level of about 30 ng/L (0.06 μM). It has been documented that when used in doses recommended for the treatment of acute malaria and malaria chemoprophylaxis, peak serum levels are about 162 ng/ml (0.31 μM) and 36 ng/ml (0.07 μM), respectively (Ritschel *et al.*, 1978). However, in the long-term use, such as in the management of connective tissue disorders, peak levels reach 800 ng/ml (1.5 μM) (Mackenzie, 1983a).

1.3.1 VOLUME OF DISTRIBUTION OF CHLOROQUINE IN BLOOD

In humans, the drug is extensively distributed with a volume of distribution of 200-800 kg/L (0.39-1.5 μM) when calculated from plasma concentration and 200 kg/L (0.39μM) when estimated from whole blood data. The concentration in whole blood is estimated to be 5 to 10 times higher than in plasma (Ducharme and Farinotti, 1996).

Chloroquine is immediately absorbed from the blood after its administration and strongly bound to tissue proteins. About 60% of it binds to both albumen and alpha-glycoprotein. It is equally bound to pigmented tissues as well as muscles and mononuclear cells. The drug is also rapidly removed from the blood to be concentrated in the liver, spleen, lungs, kidneys and all blood cells as well as the platelets. It is estimated that the concentration in the platelets reaches about 200-700 times (Gustafsson *et al.*, 1983); in the brain, it reaches 10-30 times, whereas normal erythrocytes contain about 10-20 times that in plasma. Also, parasitised erythrocytes have as much as 25 times that found in normal ones (Berliner *et al.*, 1948). The accumulation of the drug in the blood components means that haemolysis even when limited, can extensively raise measured plasma concentrations of the drug.

Since nearly 70% of the drug in plasma is found bound to non-diffused plasma constituents the situation explains why whole blood concentrations are substantially higher than their corresponding serum and plasma concentrations (Berliner *et al.*, 1948). This complex pharmacological phenomenon exhibited by chloroquine in adults and children alike, makes it imperative for the plasma level of chloroquine shortly after dosing to be determined primarily by the rate of distribution rather than elimination

(Krishna and White, 1996). This extensive tissue binding, they further intimated, requires a loading dose to achieve effective concentrations in plasma and blood.

1.3.3 METABOLISM

Within 12 hours following both oral and parenteral administration in humans, 30-50% of the absorbed chloroquine is principally biotransformed in the liver and other tissues. It is rapidly de-alkylated via cytochrome P450 enzymes (CYP) into the pharmacologically active desethyl-chloroquine (DCQ) and bisdesethyl-chloroquine (BDCQ). These metabolites reach blood concentrations of 40% and 10% of chloroquine concentrations, respectively (Ducharme and Farinotti, 1996).

1.3.4 EXCRETION

Chloroquine concentrations in humans decline multi-exponentially. After its metabolism by the liver, the rest is excreted rather unchanged as follows:- 8%-12% in faeces and about 5% through the skin (Bergqvist and Domeiji–Nyberg, 1983). The renal clearance of chloroquine is approximately half of its total systemic clearance with unchanged chloroquine and desethylchloroquine, its major metabolite, accounting for more than 50% and 30% of the urinary drug, respectively. The process of renal excretion is found to increase by acidification and decrease by alkalinisation. It is therefore worthy of note that users with renal

insufficiency and impaired hepatic functions stand a greater risk of most of the side effects of the drug (Krishna and White, 1996).

1.3.5 HALF-LIFE

Both chloroquine and desethyl-chloroquine decline slowly with average elimination half-lives of between 20 and 60 days. This rate of elimination makes it possible for traces of the drug to be detected in most body tissues as well as the urine even several months after a single dose has been administered (Furst, 1996).

1.4 MODE OF ACTION OF CHLOROQUINE

The mode of action of chloroquine is discussed here under its anti-malarial and anti-rheumatoid actions.

1.4.1 ANTI-MALARIAL ACTION

Chloroquine is found to be effective against all forms of the malarial parasites except the resistant strains of *Plasmodium falciparum* and *Plasmodium ovale* (Salako and Aderounma, 1987). Although an effective blood schizontocide, the exact primary mechanism of its anti-malarial action, like any of its related aminoquinoline drugs, somehow, remains

obscure. An earlier postulate (O'Brien *et al.*, 1966; Ciak and Hahn, 1966) suggests that chloroquine acts by intercalating between plasmodial DNA base pairs thereby interfering with nucleic acid syntheses. This assertion according to Chou *et al.* (1980), however, seems unlikely because of the rapid onset of morphological changes that are induced by low concentrations of the drug in plasmodial cells studied in infected erythrocytes. These morphological changes could not have been possible without the ability of the plasmodial parasites to undergo any form of nucleic acid syntheses leading to organelle duplication and replication suggestive that the drug might not entirely stop the process of nucleic acid syntheses and replication (Chou *et al.*, 1980). These authors suggested that the partial processes of duplication and replication, possibly, contribute partly to the addition of new protoplasm thereby enhancing the observed elaborate and rapid morphological transformations in the cells under such circumstances.

A more recent hypothesis, on the other hand, suggests that ferriprotoporphyrin IX (haemin) released during the degradation of haemoglobin in parasitised erythrocytes, serves as a receptor for chloroquine and other related anti-malarial alkaloids (Bray *et al.*, 1999). These resultant drug-haemin complexes have earlier been identified to have deleterious effects on membranous structures of plasmodia and erythrocytes (Chou *et al.*, 1980). However, earlier studies of interactions between haemin and a series of such anti-malarials including chloroquine,

by Aikawa, (1972), Warhurst, (1987) and Schlesinger *et al.*, (1988) do not fully support the above concept proposed by Bray *et al.* (1999).

Another view is that, in part, because chloroquine is a weak base, it concentrates in and raises the pH of acidic vesicles (Ginsburg and Geary, 1987) within sensitive malarial parasites. Here, the drug interferes with the lysosomal degradation of haemoglobin (Mackenzie, 1983b) and causes characteristic histological abnormalities including chloroquine-induced clumping of pigments or delayed pigmental changes (Warhurst, 1987; Schlesinger *et al.*, 1988).

For now, though the molecular mechanisms by which chloroquine and other related blood schizontocides act are unclear, inhibition of the functions of Ca^{2+}-dependent proteins such as calmodulin has been implicated (Scherbel *et al.*, 1957). Also, Read *et al.* (1999), reported that the drug interacts specifically with the glycolytic enzyme, lactate dehydrogenase from the parasite within the NADH-binding pocket of the enzyme, thereby occupying a position similar to that of the adenyl ring of the co-factor. This interaction, therefore, leads to a competition with the latter for binding to the enzyme hence acting as a competitive inhibitor for this critical glycolytic enzyme.

Finally, like many other anti-malarial quinolines, chloroquine is believed to inhibit haemoglobin proteolysis by intra-erythrocytic *Plasmodium*

falciparum. The chloroquine does this inhibition by associating with the free haem polymer in the presence of free haem molecules. It was accordingly proposed that the formed chloroquine-haem complex caps the growing polymer thereby preventing further sequestration of any additional haem molecules. Consequently, the accumulation of the haem builds up to toxic levels, finally incapacitates and subsequently kills the parasite (Sullivan *et al.*, 1998).

1.4.2 ANTI-RHEUMATOID ACTION

The anti-rheumatoid effects of chloroquine can be attributed to both its anti-inflammatory and immuno-suppressive effects (Homewood *et al.*, 1972). These properties have been adequately demonstrated *in vitro* and *in vivo*. According to Warhurst (1987) and Schlesinger *et al.* (1988), the accumulation of the drug in lysosomes contributes to its anti-rheumatoid action. This action, they observed, is due to the drug's adverse effects on acidic vesicles as indicated under its anti-malarial action above.

Chloroquine is reported to inhibit the release of cytokine and the antigen-processing functions of monocytes and macrophages (Lee *et al.*, 1982; Salmeron and Lipsky, 1983). The inhibition caused by the drug diminishes the tendencies for the formation of the peptide-MHC protein complexes required to stimulate $CD4^+$ T-cells thereby resulting in the down-regulation of the immune response mechanisms of the affected bodies (Ginsburg and Geary, 1987). It has also been shown recently that chloroquine suppresses

the ability of monocytes to produce interleukin-1 and interleukin-6 probably at the post-transcriptional level (Barrera *et al.*, 1996). Again, the drug is found to inhibit chemotaxis, phagocytoses and the production of superoxide by polymorphs and monocytes (Hurst *et al.*, 1987).

In vivo experimentations have also amply demonstrated an inhibitory effect of the drug on T-cell-dependent reactions such as delayed-type hypersensitivity reaction (Phadke *et al.*, 1982). Finally, another study has suggested an inhibition of cartilage-induced interleukin-1 by chloroquine (Barrera *et al.*, 1996).

It may therefore, be deduced from its mode of action that chloroquine has the potential of exhibiting biochemical, anti-inflammatory and immunological properties when administered.

1.5 SIDE EFFECTS/TOXICITY

The adverse effects resulting from high serum concentrations of chloroquine in the management of systemic lupus erythematosus and rheumatoid arthritis have been well documented (Ginsburg and Geary 1987). It has been suggested that there could be a relationship in the dose and blood concentration, and the side effects of the drug (Frisk-Holmberg *et al.*, 1979).

The documented side effects of chloroquine are many and varied. They may be present as one or a combination of gastrointestinal, dermatologic, neurologic, haematologic, ophthalmic, cardiovascular or musculoskeletal disabilities. The reported gastrointestinal side effects may range from nausea, vomiting and epigastric pains, through anorexia to diarrhoea (Maksymowych and Russel, 1987).

Dermatologic-related abnormalities include greying or bleaching of the hair, appearance of bluish-black oval macules, melanisation of the skin, exacerbation of psoariasis and pigmentation of nails and the palate. These pigmentary abnormalities were found on light-exposed areas in 10-20% of patients (Tuffanelli *et al.* 1963).

Neurologic disturbances catalogued include headaches, dizziness, insomnia and irritability (Bagnall, 1957). Depression of skeletal muscle contractility (Argov and Mastaglia, 1979), appearance and aggravation of muscle weakness or myopathy have also been observed (Avinazubieta and Johnson, 1995).

Cardiovascular and haematological abnormalities reported are agranulocytosis (Polano *et al.*, 1965), aplastic anaemia (Nagaratnam *et al.*, 1978), ECG changes and cardiomyopathy (Magnussen and Fine, 1977),

precipitation of porphyria (Baler, 1976), and a significant reduction in creatinine clearance (Landewe and Vergouwen, 1995).

Although there are indications of ototoxic potentials associated with the long-term administration of chloroquine, they have been found to be reversible after the withdrawal of the drug (Bernhard, 1985). There have also been reports of the occurrence of ocular side effects in addition to retinopathy, which also were reversible after withdrawal. Ocular anomalies reported include corneal deposition, diplopia and defective accommodation and convergence (Percival and Meanock, 1968). The danger posed by chloroquine may be attributed to the higher tissue levels found in humans (Maksymowych and Russel, 1987).

Based on the frequency of patients exhibiting these side effects during the usage of chloroquine for a relatively long time, it was found that it is less safe than the other aminoquinoline compounds, such as hydroxychloroquine, chloroguanide, quinacrine pamaquine and pentaquine (Percival and Meanock, 1968; Finbloom *et al.*, 1985).

1.6 EMBRYOTOXICITY

Whereas chloroquine is known to cross the placental barrier (Lindquist and Ullberg, 1972; Dencker *et al.*, 1975; Essien and Afamefuna, 1982), clinical evidence of its teratogenecity is inconclusive. Earlier reports suggested that it caused otic and ocular abnormalities in offspring of women who

used the drug for a long time during pregnancy (Hart and Naunton, 1964; Paufique and Magnard, 1969). However, subsequent studies of women who used the drug for either chemosuppression, treatment of malaria or even for the management of systemic lupus erythematosus and rheumatoid arthritis immediately before and during pregnancy failed to confirm these observations (Mackenzie 1983a,b; Wolfe and Codero, 1985; Parke, 1988; Levy *et al.*, 1991).

In the absence of any large-scale epidemiological studies in humans to establish the purported teratogenicity of chloroquine, *in vivo* (Udalova, 1967; Engmann and Vartanian, 1974; Sharma and Rawat, 1989) and *in vitro* (Ambroso and Harris, 1993) studies were done on rats.

Engmann and Vartanian (1974) subjected mature female Wistar rats to daily injections of chloroquine sulphate. Dosages of 25 mg/kg or 12.5 mg/kg body weight were administered to the animals from seven days before mating, to twenty days after a suspected pregnancy. Results from the study showed that the drug caused abortion, growth retardation, dysmorphogenesis and even embryonic and foetal death.

In their *in vitro* study, Ambroso and Harris (1993) exposed 10-day-old Sprague-Dawley rat conceptuses to culture media containing chloroquine concentrations of 10 μM (5,333 ng/ml), 20 μM (10,666 ng/ml) and 30 μM (15,999 ng/ml) for 26 hours. They established dose-related decreases in embryonic crown-rump length and visceral yolk diameter as well as

reductions in total protein and DNA contents of the embryos. Dose-related increases in the incidence of morphological abnormalities in the embryos such as microophthalmia, abnormal axial rotation and selective cephalic hypoplasia were also reported.

Studies in humans using a single oral dose of 620 mg chloroquine base (10-12 mg/kg body weight) produced a whole blood peak concentration of 1,058-1,113 ng/ml (Adjepon-Yamoah et al., 1986). Furthermore, it has been reported that in the long-term use of the drug, serum levels do not exceed 1.5 μM or 800ng/ml (Mackenzie, 1983a,b). It is therefore obvious that the dosages (12.5 and 25.0 mg/kg body weight) administered by Engmann and Vartanian (1974) and the period of administration would have resulted in accumulation of the drug in the animals to excessive levels. Similarly, the dosages applied by Ambroso and Harris (1993) (10 μM or 5,333 ng/ml, 20 μM or 10,666 ng/ml and 30 μM or 15,999 ng/ml) were clearly too high.

Hence, it may be concluded that the long period of exposure by Engmann and Vartanian (1974) and the high concentrations of the drug applied by Ambroso and Harris (1993) to the embryos, foetuses and conceptuses in their respective studies, make the interpretation of their findings difficult.

Consequently, an attempt was made to address the above issues and other related ones in a more recent *in vitro* experiment on rats by Tagoe and Ofori-Adjei (1995). Using the whole-embryo culture technique (New,

1978), $9^1/_2$-day-old post-implantation Wistar rat conceptuses were exposed to commercially available chloroquine phosphate and its enantiomers at a concentration of 500 ng/ml (0.97 μM) of culture medium. The concentration applied here, they stated, falls within the range of serum levels attained during long-term chloroquine therapy (Mackenzie, 1983a, b). The conceptuses were assessed by the morphological scoring system of Brown and Fabro (1981).

The findings of Tagoe and Ofori-Adjei (1995) suggested that the commercially available form of the drug is both embryotoxic and dysmorphogenic, even at relatively low concentration. Other findings that came out of their research were the fact that the (+)-CQ enantiomer was less embryotoxic than the (-)-enantiomer which was in turn, less so than the racemate. Abnormalities they observed included axial rotation, cranial neural tube defects (unfused and under-developed), microophthalmia and abnormal otic primordia. These authors also recorded decreases in the yolk sac diameter, crown-rump length, and the number of somites as well as the total protein contents of the embryos.

The findings by Tagoe and Ofori-Adjei (1995) have, to some extent, addressed most of the problems that characterised earlier experiments cited above. For instance, they have been able to establish a well-defined serum concentration of chloroquine in relation to levels that are attained during the long-term therapy with the drug in humans. They have also demonstrated that the corresponding serum concentration of the drug they

used in their *in vivo* study has been responsible for the abnormalities and dysmorphogenesis observed in the rat conceptuses and embryos by other authors (Engmann and Vartanian, 1974; Ambroso and Harris, 1993). Hence, it may be argued that the potential of chloroquine to cause embryonic and foetal abnormalities in humans after its long-term use, as indicated by earlier investigators has at least, been established.

As a follow up to Tagoe and Ofori-Adjei (1995), the present investigation attempts an *in vivo* assessment of the teratogenecity of chloroquine considering the important role it plays, especially, in the treatment of malaria and the management of connective tissue disorders. Furthermore, results derived from this study may serve as a very useful guide for future investigations into *in vivo* experimentations in the field of therapeutic drug assessment.

1.7 AIMS AND OBJECTIVES

The general objectives of this study are therefore to;

1. Screen five dosages of racemic chloroquine phosphate, which have been reported to cause abnormalities or deformities, especially in rat conceptuses and embryos to find out which ones will produce peak whole blood concentrations comparable to those attainable during the long-term therapeutic use of the drug.

2. Evaluate the embryotoxic and dysmorphogenic potentials of the selected dosages on $9^1/_2$ day-old conceptuses in the Sprague-Dawley rat over a 48-hour period.

3. Establish the dosage(s) and corresponding whole blood concentration of the racemic chloroquine selected that will have the least embryotoxic and dysmorphogenic potential(s).

4. Compare the results of the study with earlier ones.

CHAPTER 2

MATERIALS AND METHODS

The materials and methods consisted of the following three components:

1. **PHARMACOKINETIC STUDIES**
2. **EMBRYOTOXICITY STUDIES**
3. **TOTAL PROTEIN CONTENT DETERMINATION**

2.1PHARMACOKINETIC STUDIES

These studies were in two parts involving;

(i) Non-pregnant female rats

(ii) Pregnant rats

2.1.1RATIONALE

The rationale for the pharmacokinetic studies was to;

(i) Determine the dosages of chloroquine diphosphate, which will provide blood levels in the non-pregnant Sprague-Dawley rats comparable to those achievable during therapeutic use of the drug. The accepted dosages will then be used for the embryotoxic studies. The dosages selected were 7.5 mg/kg body weight, 10.0 mg/kg, 12.5 mg/kg body weight, 15.0 mg/kg body weight and 20.0 mg/kg body weight. These dosages were carefully chosen based on the results from a series of *in vivo* (Engmann and Vartanian,

1976) and *in vitro* (Ambroso and Harris, 1993; Tagoe and Ofori-Adjei, 1995) investigations in rats. Similar *in vivo* experiments in humans by Gustafsson *et al.* (1983) and Adjepon-Yamoah *et al.* (1986) were also considered.

Engmann and Vartanian (1976) used 12.5 mg/kg body weight or 25 mg/kg body weight on pregnant rats for various lengths of time and induced embryonic and foetal deaths. Similarly, Gustafsson *et al.* (1983) recorded whole blood concentration levels of chloroquine in adult humans ranging from 94 ng/ml to 120 ng/ml. This was after a single oral dose of 300 mg chloroquine phosphate (approximately 5-6 mg/kg body weight) was administered to subjects.

Adjepon-Yamoah *et al.* (1986) did a subsequent human single-dose chloroquine kinetics determination study and recorded peak whole blood chloroquine concentration levels ranging from 1,058 ng/ml to 1,313 ng/ml. This was after an oral administration of a single dose of 620 mg chloroquine base (approximately 10-12 mg/kg-body weight), barely double the dose employed by Gustafsson *et al.* (1983).

(ii) Analyse blood samples from pregnant rats on gestation day $11^1/_2$ for the presence of chloroquine, after they were injected with the

selected dosages on gestation day $9^1/_2$. Blood samples were taken just at the time they were being sacrificed to ascertain the presence or otherwise of chloroquine in them. The samples were taken only at the time of sacrifice because taking samples as done for the non-pregnant ones might subject the animals to a stressful condition that will adversely affect the results or even induce abortion. This was particularly due to the painful method of acquiring blood from the coccygeal vein, which inevitably inflicted some physical and physiological stresses on the animals as described under section 2.1.5 below.

2.1.2 THE DRUG CHLOROQUINE

The commercial racemic chloroquine (CQ) diphosphate of purity 97% was obtained from ACO (Solna, Sweden). A solution of the drug was prepared in 0.15 M phosphate–buffered saline (PBS) at pH = 7.4, from a stock solution of 4 mg/100 ml of original concentration.

2.1.3THE EXPERIMENTAL ANIMALS

Twenty healthy female Sprague-Dawley rats of ages ranging from eight to ten weeks and weighing between 200 and 225 g were obtained from the Laboratory Animals Unit of the Noguchi Memorial Institute for Medical Research (NMIMR), College of Health Sciences (CHS), University of Ghana, Legon, Accra. The animals were transported from the NMIMR to the Centre for Tropical Clinical Pharmacology and Therapeutics

(CTCPT), Korle-bu, in metal cages stuffed with wood shavings. They were supplied with water and normal commercially prepared rodent feed formulated by the Laboratory Animals Unit of the NMIMR throughout the period. The journey lasted for about one hour forty five minutes. A period of two days was allowed for the animals to recover from a possible physiological stress during the journey before the commencement of the experiment.

2.1.4DRUG ADMINISTRATION/INJECTION

The animals were injected intraperitoneally with the five dosage regimens of the chloroquine diphosphate selected. The injection was done using a 1.0 ml capacity syringe and a fine needle of aperture 0.16 mm.

2.1.5 COLLECTION OF BLOOD SAMPLES

Blood collection was done by carefully cutting the tip of the tail of each animal after cleaning the site with 70% ethyl alcohol. This was followed by gently squeezing, from the tail base to the tip, for blood through the coccygeal vein into 70 microlitre micro haematocrit heparinised (capillary) tubes. The contents were dissolved into 1 ml of 0.1% diethylamine solution in 5 ml capacity glass tubes, covered with a screw cap and frozen. Each sample was collected in duplicates. The animals were continuously fed on their normal diet of rodent feed and given water during the experimentation period to ensure the continuous flow of blood

and also minimise any possible stress which may adversely affect the animals and hence, the results.

Collection of blood samples was done as follows: -

- For non-pregnant female rats, blood samples were taken at pre-dose, 0.25 hr, 0.50 hr, 0.75 hr, 1.25 hr, 1.75 hr, 2.75 hr, 13.50 hr, 19.50 hr, 22.00 hr and 24.00 hr after the administration of the drug.
- Blood samples were taken from pregnant rats just at the time of being sacrificed on day $11^1/_2$ of gestation. (See 2.1.1 ii above for reasons).

2.1.6 ANALYSIS OF BLOOD SAMPLES (CHLOROQUINE ASSAY)

The blood samples collected were analysed by the High Performance Liquid Chromatography (HPLC) method. The solvents and other chemicals and reagents used were of analytical grade and used as specified by the manufacturers (Aldrich-Chemie, Steinheim, Germany and Sigma Chemical Company, St. Louis, Mo., U.S.A.). The samples were analysed by the following processes;-

Quantities of 75 ml dihydro-quinidine as internal standard (IS), 0.5 ml of 1.0 M NaOH, and 6 ml of diethylether were added to the blood samples. The whole mixture was then mixed with a Whirlmix spiral mechanical mixer for 15 minutes and centrifuged for 10 minutes at 8000 g. The etheral layer was pipetted off into glass extraction tubes and evaporated in a water bath at a temperature of between 37°C and 40°C. The extract was

reconstituted using 200 ml of the mobile phase after which 50 ml of it was injected into the HPLC system. Standard curves were obtained alongside the samples by using five different concentrations of chloroquine and its major metabolite, desethylchloroquine (DCQ). (see Appendix A).

The chromatographic separation was carried out on a LKB (Broma) 2150 HPLC pump and monitor at a flow rate of 1.0 ml/minute using a straight phase column and a Rheodyne injector. The detector was a SHIMAZU RF-530 Fluorescence model, operated at wavelengths of 385 nm and 335 nm for emission and extinction, respectively.

2.1.6.1 STANDARD CURVE

A standard curve was drawn using chloroquine concentrations of 0, 100, 200, 400, 600 and 800 ng/ml as indicated in Appendix A.

2.2 EMBRYOTOXICITY STUDIES

2.2.1 MATING OF RATS

One each of healthy adult male and female rats were put together in metal cages with solid floors stuffed with wood shavings overnight. This was done in an air-conditioned room of temperature measuring 18°C to 22°C with relative humidity range of 50% to 70%. The cages measured 250 cm^3. A ratio of 12:12 light: darkness cycle was also provided in the room. The animals were given water and fed on their normal pellet diet formulated by the Laboratory Animals Unit of the NMIMR. The cages

were examined for vaginal plugs every morning at 09.00 hr GMT, the presence of which signified coitus and a possible pregnancy. The midnight prior to the observation of the plug was designated day zero. Immediately the vaginal plugs were detected, the male rat was removed and the female kept alone to avoid a possible tussle that may lead to an abortion or any form of incapacitation on the part of the female.

2.2.2 INJECTION OF CHLOROQUINE

Out of the five dosages screened in the pharmacokinetic studies, the lower three (7.0 mg/kg body weight, 10.0 mg/kg body weight and 12.5 mg/kg body weight) were selected to be used for the embryotoxicity studies. This was because the dosages produced whole blood peak concentrations that fall within the range attainable during long-term therapy with the drug. The drug was administered intraperitoneally on conception day $9^1/_2$ at approximately 09:00 hr GMT using a fine 0.16 mm aperture needle.

The injection of chloroquine diphosphate involving the three dosage regimens were as follows:-

(i) 7.5 mg/kg body weight of CQ constituted in 0.4 ml PBS.
Six pregnant rats were used for this treatment group.

(ii) 10.0 mg/kg body weight of CQ constituted in 0.4 ml PBS.
Eight pregnant rats were used for this treatment group.

(iii) 12.5 mg/kg body weight of CQ constituted in 0.4 ml PBS.

Seven pregnant rats were used for this treatment group.

(iv) Control rats were injected with 0.4 ml PBS only.

In all, eight pregnant rats were used for this treatment group.

2.2.3 EXPLANTATION OF EMBRYOS

Explantation of the embryos was done just after blood samples were taken from the pregnant rats for analysis. Exposure of the embryos was done after anaesthetising each rat by injecting it intraperitoneally with phenorbarbitol at a dosage of 20 mg/kg body weight. The unconscious rat was then placed supine on a dissection board with the four limbs spread and held tautly apart with dissection pins through the hands and feet.

Afterwards, the abdominal hair was wetted with cotton wool drenched in ethyl alcohol and a midline incision made through the abdominal wall from the anus to the xiphoid cartilage, long enough to expose both uterine horns as in Figure 2. Further transverse cuts were made on both sides midway between the xiphoid cartilage and the anus for easy access to the uterine horns.

Each uterine horn was carefully examined after which the number and uterine sites were recorded (Figure 3). The entire uterus of the rat was

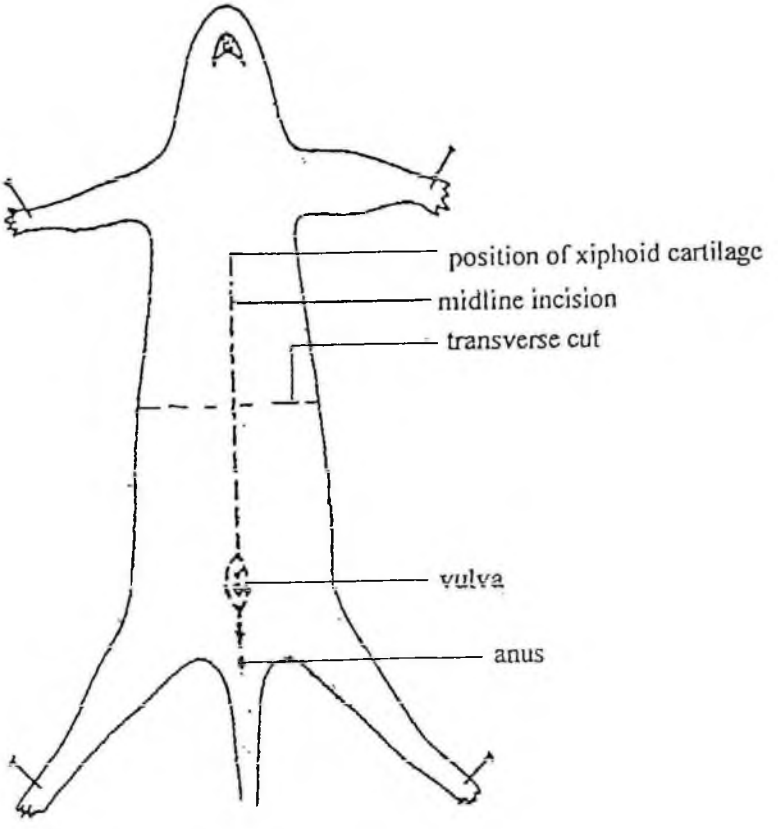


FIGURE 2:

Illustration of the incisions for the opening of the abdominal and pelvic cavities of the pregnant rats to expose the embryos.

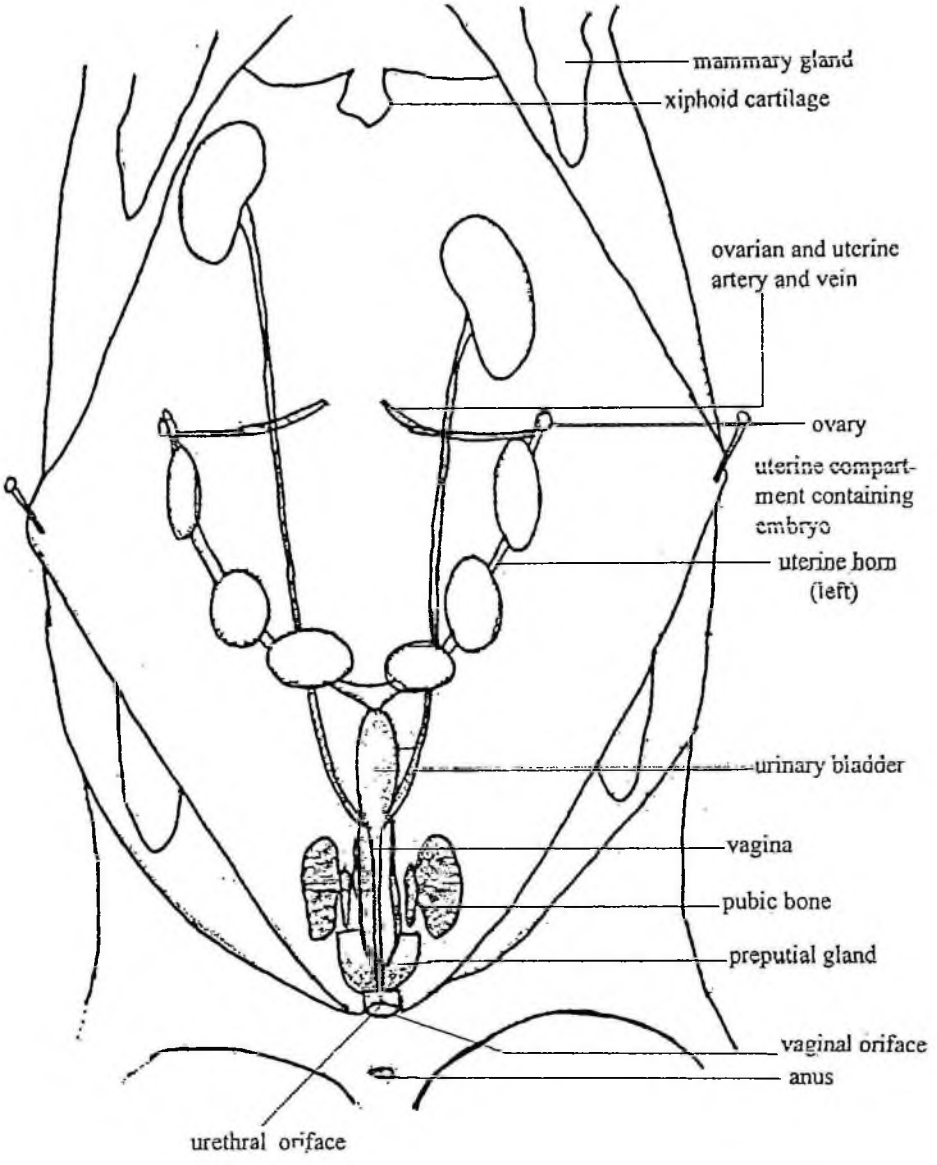


FIGURE 3:

Illustration of the dissection showing the positions of the uterine horns and some related abdominal and pelvic viscera at the final stage of the dissection of the pregnant rats just before the removal of the entire uterus containing the embryos.

removed and then transferred into a petri dish of phosphate-buffered saline solution of pH=7.4. The individual foetal compartments were cut out along the entire length of the uterus. Each compartment was then carefully torn open with a pair of fine forceps thereby exposing the pear-shaped mass of decidual tissue from which the embryo and its membranes were dissected out.

2.3 MORPHOLOGICAL ASSESSMENT

The morphological assessment of the dissected embryos was done using a Wild M8 dissecting microscope. Embryos to be assessed were selected at random in order to minimise any form of bias. Photographs were then taken of the relevant embryos. The assessment was in two parts as follows: -

(1) MORPHOLOGICAL SCORING

(2) MEASUREMENT OF GROWTH PARAMETERS.

2.3.1 MORPHOLOGICAL SCORING

This was based on the principle of morphological scoring of rodent embryos developed by Brown and Fabro (1981), using Sprague-Dawley rat embryos of 10-13 days gestation (See Table 1). This method employed a system whereby 17 features involving 6 developmental stages are scored. The stages considered are related to the following embryonic and foetal structures and systems;-

(i) Circulation; - comprising the yolk sac circulatory system, the allantois, and the heart.

(ii) Flexion (Orientation)

(iii) Nervous System; - comprising the caudal neural tube, hind, mid and fore brains, otic, optic and olfactory systems.

(iv) Pharyngeal apparatus;- comprising the pharyngeal bars, maxillary and mandibular processes.

(v) Limbs; -comprises the fore and hind limbs.

(vi) Somites

The features were scored from 0-5 with an increase in scores corresponding to an increase in the level of development. For instance, the yolk sac circulatory system was scored numerically zero denoting no visible or scattered islands and 1-4 showing increasing nature of extensive vitelline vasculature containing numerous pigmented red blood cells and steady circulation. Flexion also was scored from 0-4 as either being ventrally convex, turning, dorsally convex or even dorsally convex with spiral torsion.

The yolk sac was removed carefully by dissection and the measurements of the crown-rump and head lengths taken. The embryos were evaluated for neural tube closure, axial rotation and morphological abnormalities. The level of the limb development was also assessed with zero indicating

		0	1	2	3	4	5	SCORE
A	YOLK SAC CIRCULATORY SYSTEM	No visible or scattered blood islands	Corona of blood islands or with anastomoses	Vitelline vessels with few yolk sac vessels	Full yolk sac plexus of vessels	Yolk stalk obliterated. Vitelline artery and vein well separated		
B	ALLANTOIS	Allantois free in exocoelom	Allantois fused with cherion	Umbilical vessels	Separate aortic origins of umbilical and vitelline vessels			
C	FLEXION	Ventrally convex	Turning	Dorsally convex	Dorsally convex with spiral tension			
D	HEART	Endocardial rudiment not visible or visible but not beating	Beating "s-"shap -ed cardiac tube	Convoluted cardiac tube	Bulbus corda atrium commune and ventriculus commune	Dividing atrium commune		
E	CAUDAL NEURAL TUBE	Neural plate or neural folds	Closing but unfused neural folds (groove)	Neural folds fused at level somites 1-5	Posterior neuropore formed but upen	Posterior neuropore closed		
F	HIND BRAIN	Neural plate	Rhombomeres A and B	Anterior neuropore formed but open	Anterior neuropore closed rhombencephalon formed	Pronounced pontine flexure with transparent roof of 4th ventricle		
G	MID BRAIN	Neural plate	Mesencephalic brain folds	Closing or fusing mesencephalic brain folds	Completely fused mesencephalon	Visible division between mesencephalon and diencephalon		
H	FORE BRAIN	Neural plate or no visible prosence-nphalon	Prosencephalon brain folds	Completely fused prosencephalon	Visible telencephalic evaginations	Well elevated telencephalic hemispheres		
J	OTIC SYSTEM	No sign of otic development	Flattered or indented otic primordium	Otic pit	Otocyst	Otocyst with dorsal recess	Otocyst with endolymphatic duct	
K	OPTIC SYSTEM	No sign of optic development	Sulcus opticus	Elongated optic primordium	Primary optic vesicle with open optic stalk	Indented lens plate	Lens pocket or lens vesicle	
L	OLFACTORY SYSTEM	No sign of olfactory development	Olfactory plate	Olfactory plate with ridge	Distinct olfactory ridges	Lateral nasal process and medial rim		
M	BRANCHIAL BARS	None visible	I visible	I and II visible	I, II and III visible	II overgrowing and obscuring III		
N	MAXILLARY PROCESS	No sign of maxillary development	Maxillary proce-ss demarcated as visible cleft anterior to bar I	Maxillary proce-ss fused to nasal process				
P	MANDIBULAR PROCESS	No sign of mandibular development from bars I	First branchial bars fused and forming mandibular process					
Q	FORE LIMB	No sign of fore limb development	Distinct invagination of wolfian crest at levels of somite 9 – 13	Fore limb bud	Paddle - shaped fore limb bud	Distinct apical ridge on fore limb bud		
R	HIND LIMB	No sign of hind limb development	Distinct evagination of wolfian crest at level of somites 26 -30	Hind limb bud	Paddle- shaped hind limb bud			
S	SOMITES	0 – 5	7 – 15	16 – 20	21 – 27	38 – 41	35 - 41	

Morphological Scoring System. To use the score sheet, each of the 17 features (A – S) is examined and scored in a fresh sample, and the total of all scores is the morphological score. Flexion should be evaluated before opening the yolk sac and the heart is easier to examine while still containing blood. The text contains further explanation of some of the stages and Figures 6-8 illustrate many of the features and their appropriate scores. A larger copy of the score sheet for routine laboratory use is available from the authors (BROWN & FABRO, 1981).

no sign of limb development. Scoring 1-4 again, shows increases in development, to the appearance of a distinct apical ridge on the fore limb bud and a paddle shaped hind limb bud.

Finally, the individual scores of all the 17 features were pooled to give a total morphological score for each embryo. Each individual embryo was then placed in a plastic tube and frozen for the subsequent determination of the total protein content.

2.3.2 MEASUREMENT OF GROWTH PARAMETERS

The growth parameters measured were: -

1. Yolk sac diameter.
2. Head length.
3. Crown-rump length.
4. Number of somites.

2.3.2.1 YOLK SAC DIAMETER.

The measurement of the diameter of the vascular yolk sacs was taken along the widest region of the structure using an eyepiece graticule as seen from Figure (4A).

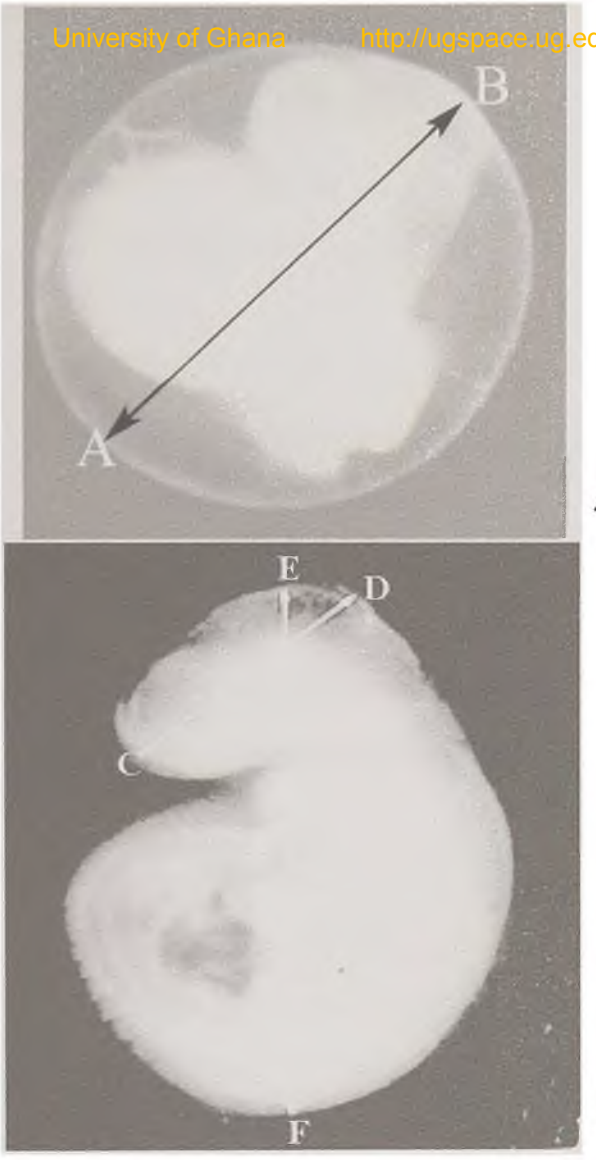


FIGURE 4:

Illustration of the measurements of the morphological parameters.

LEGEND

Distance **A-B :** Yolk sac diameter
Distance **C-D:** Head length
Distance **E-F:** Crown-rump length

2.3.2.2 HEAD LENGTH.

This measurement was done using an eyepiece graticule. This is the distance (C-D) from the dorsal hindbrain to the rostral forebrain when viewed from the side as seen from Figure 4(B).

2.3.2.3 CROWN-RUMP LENGTH.

It is the distance (E-F) from the crown to the rump (the tail base) using an eyepiece graticule as seen from Figure 4(B).

2.3.2.4 NUMBER OF SOMITES.

The number of somites for the individual embryos was determined by direct visual count with the help of the dissecting microscope.

2.4 DETERMINATION OF TOTAL PROTEIN CONTENT

This involved the determination of the total protein content of each individual embryo. This was done using a modified version of the method devised by Lowry *et al.* (1951). See Appendix B for details.

2.4.1 MODIFIED LOWRY PROTEIN ESTIMATION FOR USE IN DETERMINING EMBRYO PROTEINS IN 1.0 M NaOH

2.4.1.1 PREAMBLE

This method is primarily based on colour development of samples. The intensity of the colour produced is sensitive to pH changes. Therefore, the presence of 1.0 M NaOH in the sample shifts the reaction pH away from

its optimum. The addition of an acid neutralises the alkali. Hence, the buffers in the reagents help control the pH balance effectively.

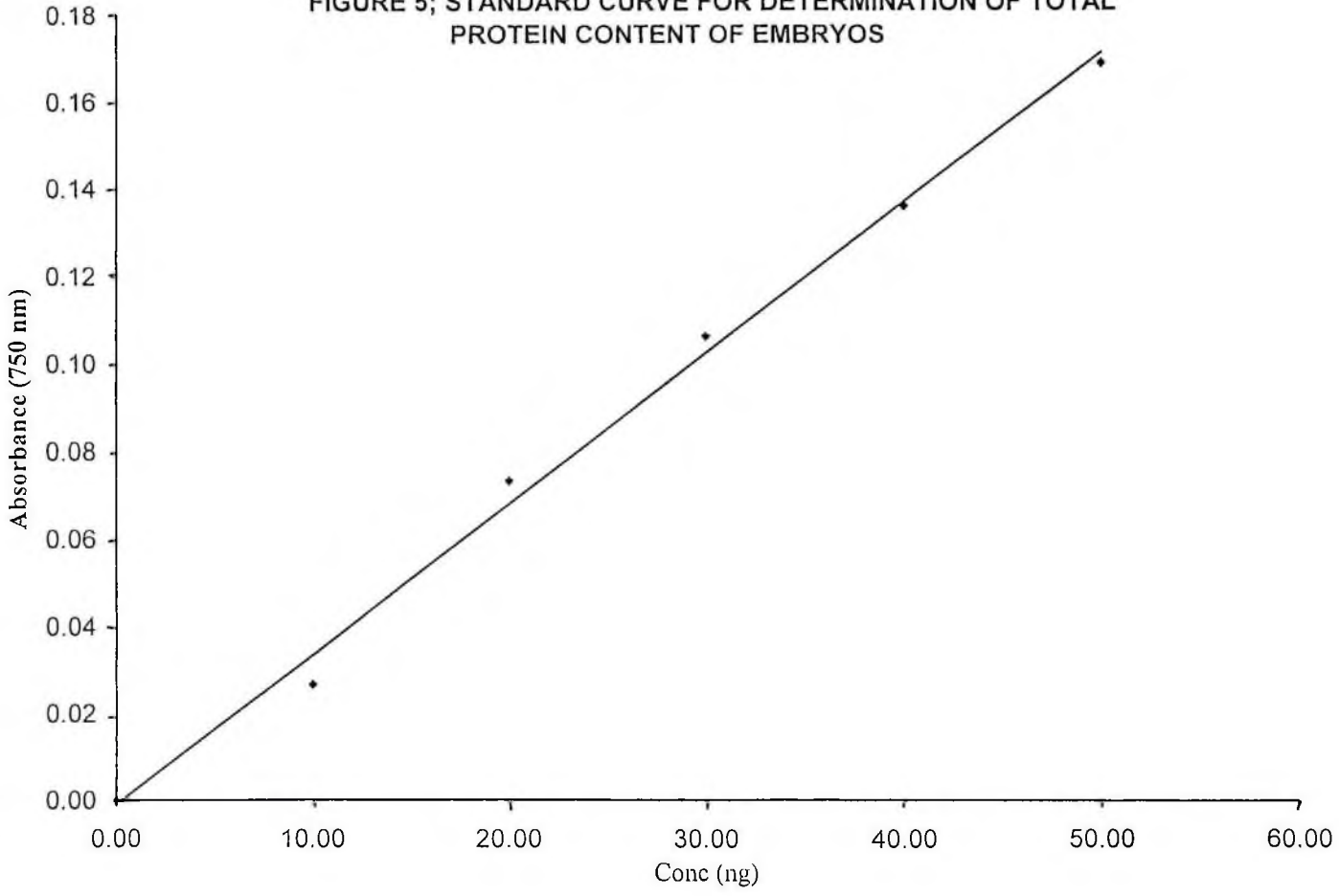
2.4.1.2 METHOD

Each embryo was placed in 1.0 ml of 1.0 M NaOH in a sealed tube and incubated at 37°C for two hours, whirl-mixing every 10-15 minutes after which 0.145 ml of 3 M HC1 was added and mixed thoroughly. 0.5 ml aliquots, two from each sample, were transferred into 9 ml plastic tubes. 2.5 ml of Folin A (See Appendix B for preparation) were added to each sample in the tube and left at room temperature for 20 minutes. Thereafter, 0.25ml of commercially prepared Folin B was added and then thoroughly MIXED IMMEDIATELY. This was necessary because any delays would diminish the colour reaction. The whole set-up was left at room temperature for 45 minutes and the absorbance at a wavelength of 750 nm was determined against a distilled water blank using a SPECTRONIC D20 (UV/VIS) spectrophotometer.

2.4.1.3 STANDARD CURVE

The standard curve for detecting the embryonic protein content was drawn by measuring the amount of protein in samples of bovine serum albumen (BSA) stock solution (4.58 mg BSA/ml dissolved in 1.0 M NaOH, stored at -20^{0}C). Volumes of the stock solution, 0.5-50 μl, were taken in duplicates. Each was then made up to 1.0 ml by adding 1.0 M NaOH. Subsequent steps were the same as in 2.4.1.2 above.

FIGURE 5; STANDARD CURVE FOR DETERMINATION OF TOTAL PROTEIN CONTENT OF EMBRYOS



The amount of protein detected in the (0.5ml) samples of the BSA ranged from 0 to 100 μg. A standard curve (Figure 5) was drawn using the table in Appendix B. From the gradient the amount of protein per embryo was calculated from the formula; **y = a + bx, as follows**:

$$\textbf{Protein content of embryo (μg)} = \frac{\textbf{c}}{\textbf{Gradient}} \textbf{ X } \textbf{2.29}$$

Where: **c** = corrected absorbance (**observed - blank**)

Observed = Absorbance of sample.

Blank = Absorbance of distilled water blank (0μl BSA in standard).

2.29 = Correction factor based on minimum sample total of **1.145 ml**, in **0.5 ml** aliquots.

2.5 STATISTICAL ANALYSES AND INTERPRETATION

Values obtained for yolk sac diameter, total protein content, crown-rump length, number of somites, and the total morphological scores were compared using a one-way analysis of variance (ANOVA) followed by the Bonferroni Multiple Comparison Test. The abnormality rates were analysed by the Fisher's exact test. Differences were accepted as significant when $P < 0.05$.

CHAPTER 3

RESULTS

3.1 PHARMACOKINETIC STUDIES

This study was in two parts involving;

(i) non-pregnant female rats

(ii) pregnant rats

3.1.1 NON-PREGNANT FEMALE RATS

3.1.1.1 INJECTION OF CHLOROQUINE AT DOSAGE OF 7.5 MG/KG BODY WEIGHT

This dosage produced a whole blood peak value of 1,125.54 ng/ml at 1.75 hr after rising from 1,017.32 ng/ml at 0.25 hr. The concentration then fell steeply to 129.87 ng/ml at 13.50 hr. Measurements from 19.50 hr showed no trace of the drug in the blood (Tables 2 and 7; and Figure 6).

3.1.1.2 INJECTION OF CHLOROQUINE AT DOSAGE OF 10.0 MG/KG BODY WEIGHT

As shown in Tables 3 and 7 and Figure 7, peak drug level of 1,688.31 ng/ml was achieved within 15 minutes (at 0.25 hr) following the injection. There was a steep fall in the concentration of the drug by 0.75 hr. There was no trace of the drug in the blood beyond 13.50 hr (Figure 7).

TABLE 2: CHLOROQUINE LEVELS IN WHOLE BLOOD OF NON-PREGNANT RAT INJECTED AT A DOSAGE OF 7.5 MG/KG BODY WEIGHT

Time(hr)	Peak Height Ratio (PHR)		Mean PHR	Conc (ng)		Conc (µM)
	A	B		Per 70µl	Per ml	
0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.26	0.21	0.24	71.21	1017.32	1.98
0.50	0.19	0.17	0.18	54.55	779.22	1.51
0.75	0.21	0.23	0.22	66.67	952.38	1.85
1.25	0.20	0.30	0.25	75.76	1082.25	2.10
1.75	0.24	0.28	0.26	78.79	1125.54	2.19
2.75	0.11	0.10	0.11	31.82	454.55	0.88
13.50	0.04	0.02	0.03	9.09	129.87	0.25
19.50	0.00	0.00	0.00	0.00	0.00	0.00
22.00	0.00	0.00	0.00	0.00	0.00	0.00
24.00	0.00	0.00	0.00	0.00	0.00	0.00

FIGURE 6: CHLOROQUINE LEVELS IN WHOLE BLOOD OF NON-PREGNANT RAT INJECTED AT DOSAGE OF 7.5 MG/KG BODY WEIGHT

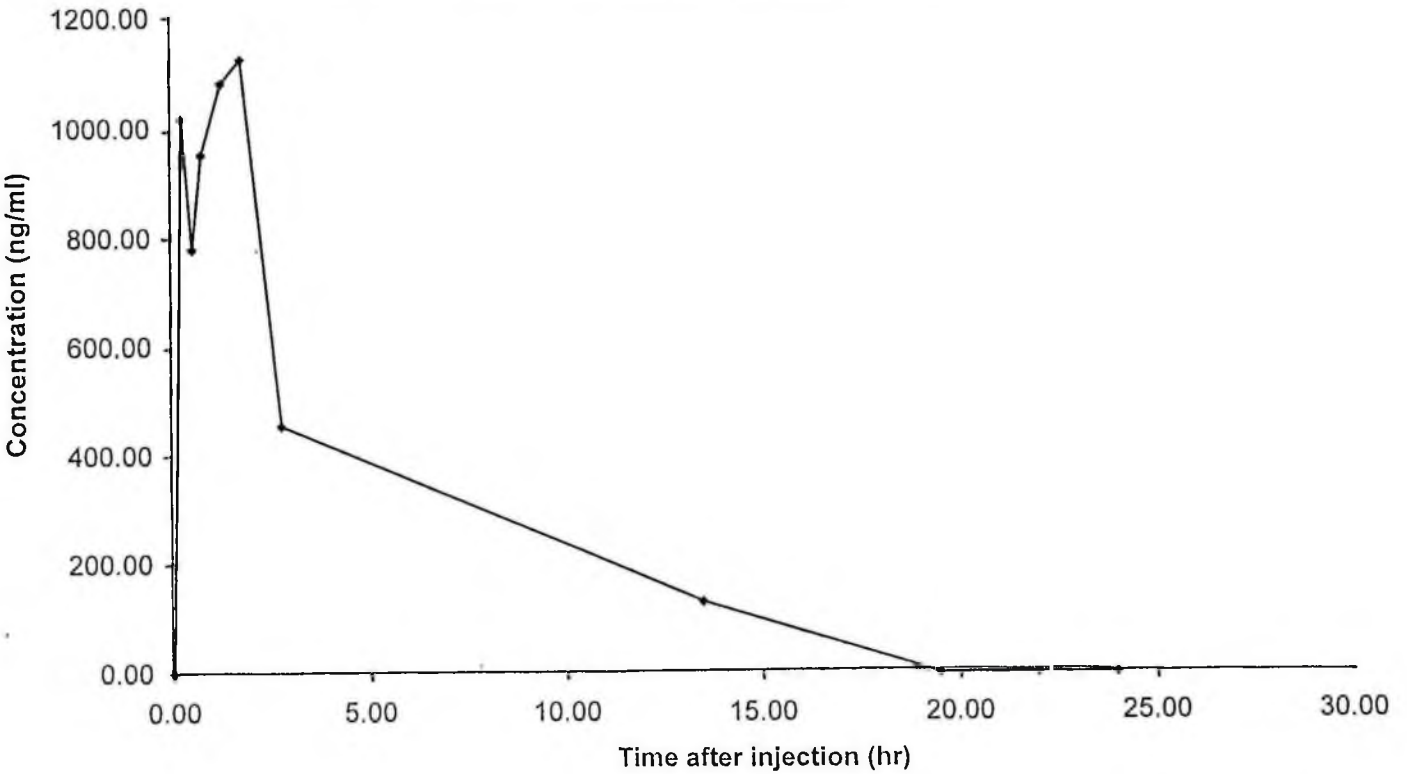
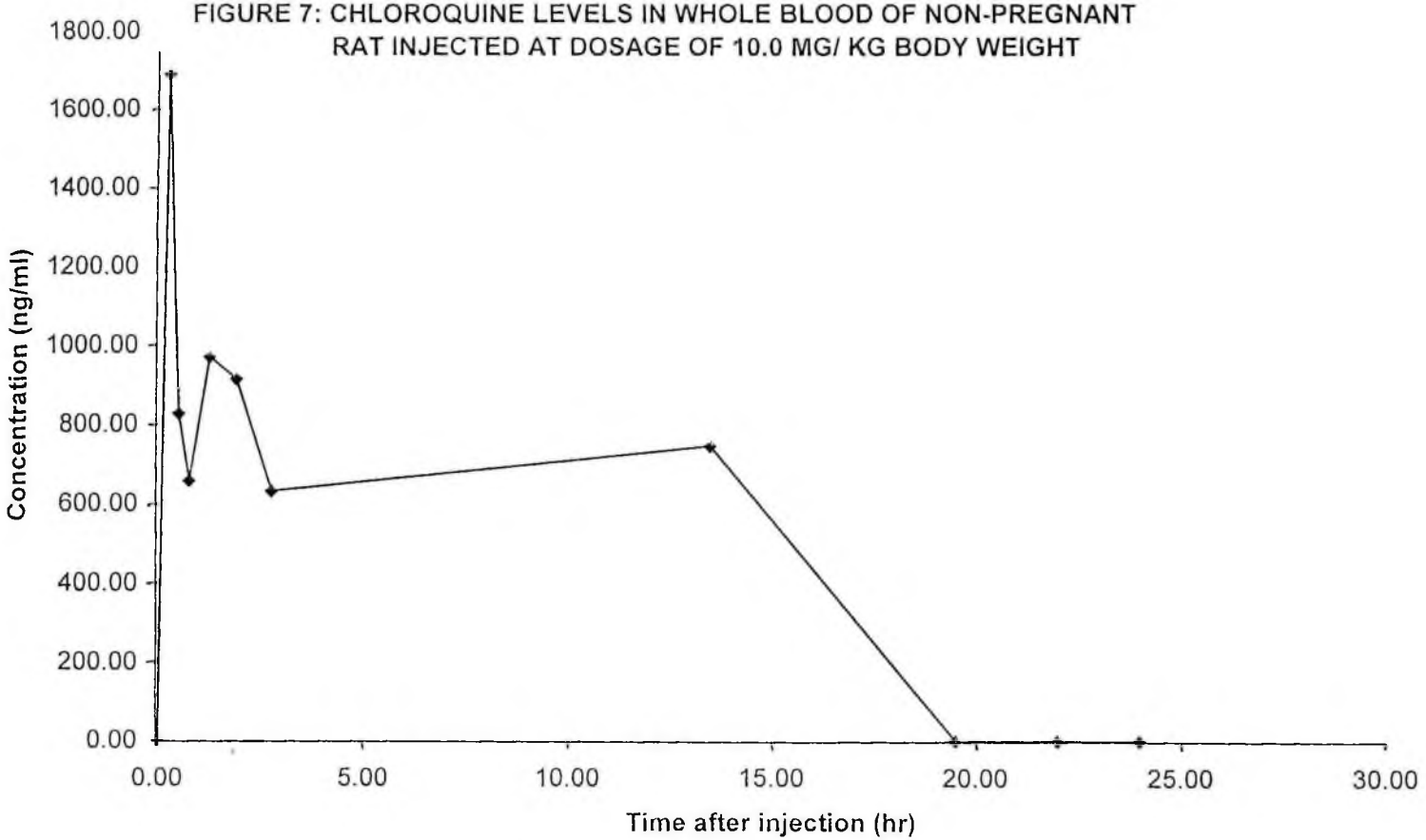


TABLE 3: CHLOROQUINE LEVELS IN WHOLE BLOOD OF NON-PREGNANT RAT INJECTED AT A DOSAGE OF 10.0 MG/KG BODY WEIGHT

Time(hr	Peak Height Ratio (PHR)		Mean PHR	Conc (ng)		Conc (µM)
	A	B		Per 70µl	Per ml	
0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.25	0.53	0.39	118.18	1688.31	3.28
0.50	0.19	0.19	0.19	57.88	826.84	1.61
0.75	0.13	0.17	0.15	46.06	658.01	1.28
1.25	0.24	0.21	0.22	67.88	969.70	1.88
1.90	0.16	0.26	0.21	63.94	913.42	1.77
2.75	0.14	0.15	0.15	44.24	632.03	1.23
13.50	0.19	0.16	0.17	52.27	746.75	1.45
19.50	0.00	0.00	0.00	0.00	0.00	0.00
22.00	0.00	0.00	0.00	0.00	0.00	0.00
24.00	0.00	0.00	0.00	0.00	0.00	0.00

FIGURE 7: CHLOROQUINE LEVELS IN WHOLE BLOOD OF NON-PREGNANT RAT INJECTED AT DOSAGE OF 10.0 MG/ KG BODY WEIGHT



3.1.1.3 INJECTION OF CHLOROQUINE AT DOSAGE OF 12.5 MG/KG BODY WEIGHT

There was a steep rise from 452.90 ng/ml at 0.25 hr to a peak level of 1811.80 ng/ml at 1.25 hr. The concentration of the drug in the blood continually declined steeply till at 19.50 hr when it reached a level of 208.60 ng/ml. Further analysis of blood samples after this time showed no drug in them (see Tables 4 and 7; and Figure 8).

3.1.1.4 INJECTION OF CHLOROQUINE AT DOSAGE OF 15.0 MG/KG BODY WEIGHT

At 0.75 hr, a peak level of 4,157.00 ng/ml was recorded after rising steeply from 1,332.90 ng/ml at 0.25 hr. The level of the drug however, fell steeply to 1,190.0 ng/ml at 1.25 hr and then gradually to 476.20 ng/ml at 19.50 hr. Blood samples collected at 22.0 hr and beyond however, recorded no chloroquine in them (see Tables 5 and 7; and Figure 9).

3.1.1.5 INJECTION OF CHLOROQUINE AT DOSAGE OF 20.0 MG/KG BODY WEIGHT

The chloroquine level in the blood rose sharply to a peak of 7,674.30 ng/ml at 0.50 hr, from a level of 3,333.30 ng/ml at 0.25 hr. It then fell rapidly to 238.30 ng/ml at 1.25 hr. It finally declined steeply to 143.00

TABLE 4 : CHLOROQUINE LEVELS IN WHOLE BLOOD OF NON-PREGNANT RAT INJECTED AT A DOSAGE OF 12.5 MG/KG BODY WEIGHT

Time(hr)	Peak Height Ratio (PHR)		Mean PHR	Conc (ng)		Conc (µM)
	A	B		Per 70µl	Per ml	
0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.12	0.14	0.13	31.70	452.90	0.90
0.50	0.38	0.30	0.34	82.93	1184.67	2.30
0.75	0.48	0.39	0.44	107.32	1532.90	3.00
1.25	0.63	0.41	0.52	126.83	1811.85	3.60
1.75	0.22	0.77	0.50	120.73	1724.74	3.40
2.75	0.24	0.28	0.26	63.41	905.92	1.80
13.50	0.16	0.19	0.18	42.68	609.76	1.20
19.50	0.09	0.03	0.06	14.63	208.60	0.40
22.00	0.00	0.00	0.00	0.00	0.00	0.00
24.00	0.00	0.00	0.00	0.00	0.00	0.00

FIGURE 8: CHLOROQUINE LEVELS IN WHOLE BLOOD OF NON-PREGNANT RAT INJECTED AT 12.5 MG/KG BODY WEIGHT

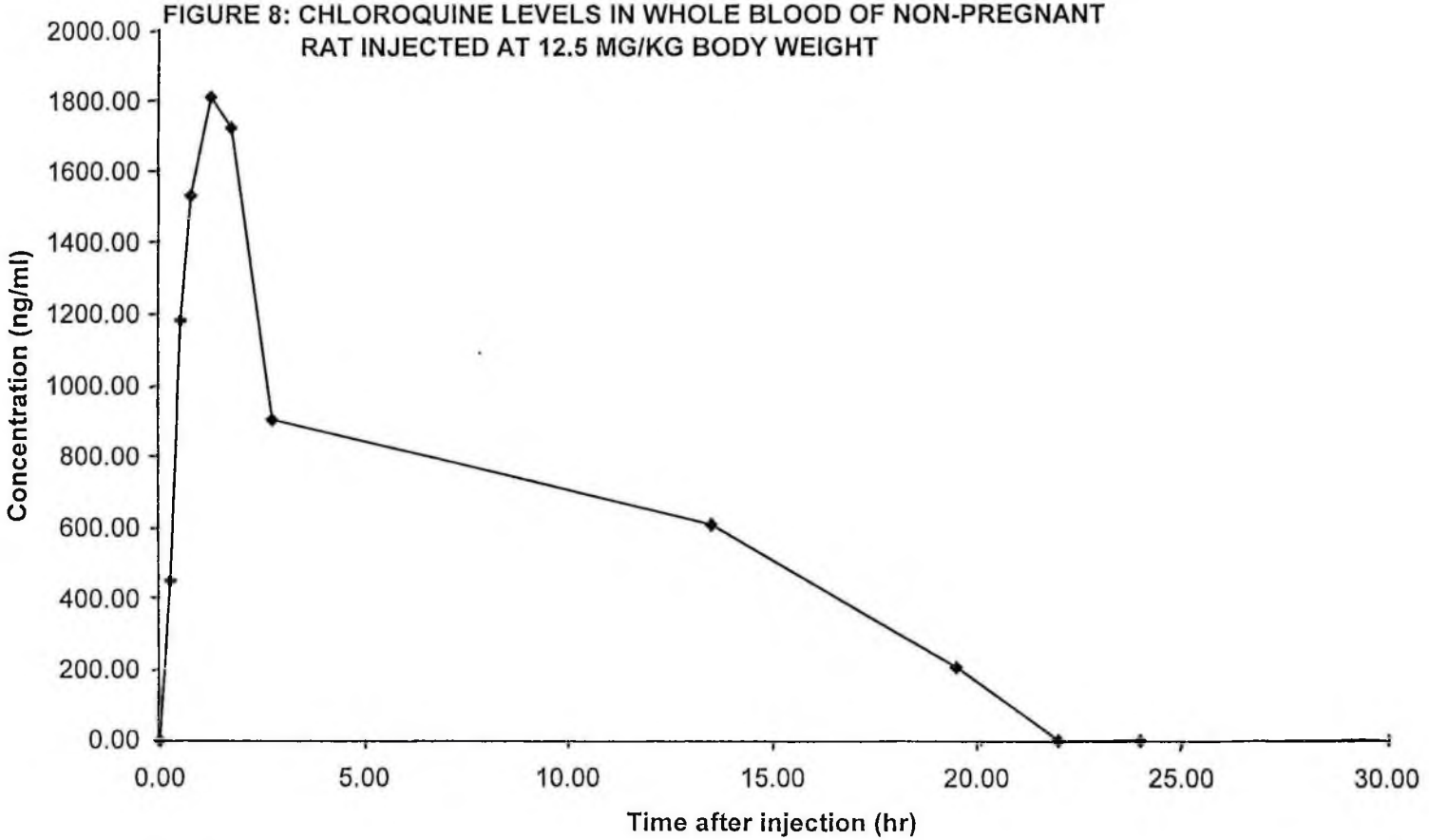


TABLE 5: CHLOROQUINE LEVELS IN WHOLE BLOOD OF NON-PREGNANT RAT INJECTED AT A DOSAGE OF 15.0 MG/KG BODY WEIGHT

Time(hr)	Peak Height Ratio (PHR)		Mean PHR	Conc (ng)		Conc (µM)
	A	B		Per 70µl	Per ml	
0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.24	0.31	0.28	93.30	1332.85	2.60
0.50	0.54	0.49	0.52	173.30	2468.42	4.90
0.75	0.41	0.43	0.42	291.30	4157.00	8.20
1.25	0.23	0.27	0.25	83.30	1190.00	2.30
1.75	0.21	0.26	0.24	80.00	1142.00	2.20
2.75	0.26	0.29	0.28	93.30	1332.86	2.60
13.50	0.20	0.18	0.19	63.30	904.29	1.80
19.50	0.09	0.11	0.10	33.33	476.18	0.90
22.00	0.00	0.00	0.00	0.00	0.00	0.00
24.00	0.00	0.00	0.00	0.00	0.00	0.00

FIGURE 9: CHLOROQUINE LEVELS IN WHOLE BLOOD OF NON-PREGNANT RAT INJECTED AT DOSAGE OF 15.0 MG/KG BODY WEIGHT

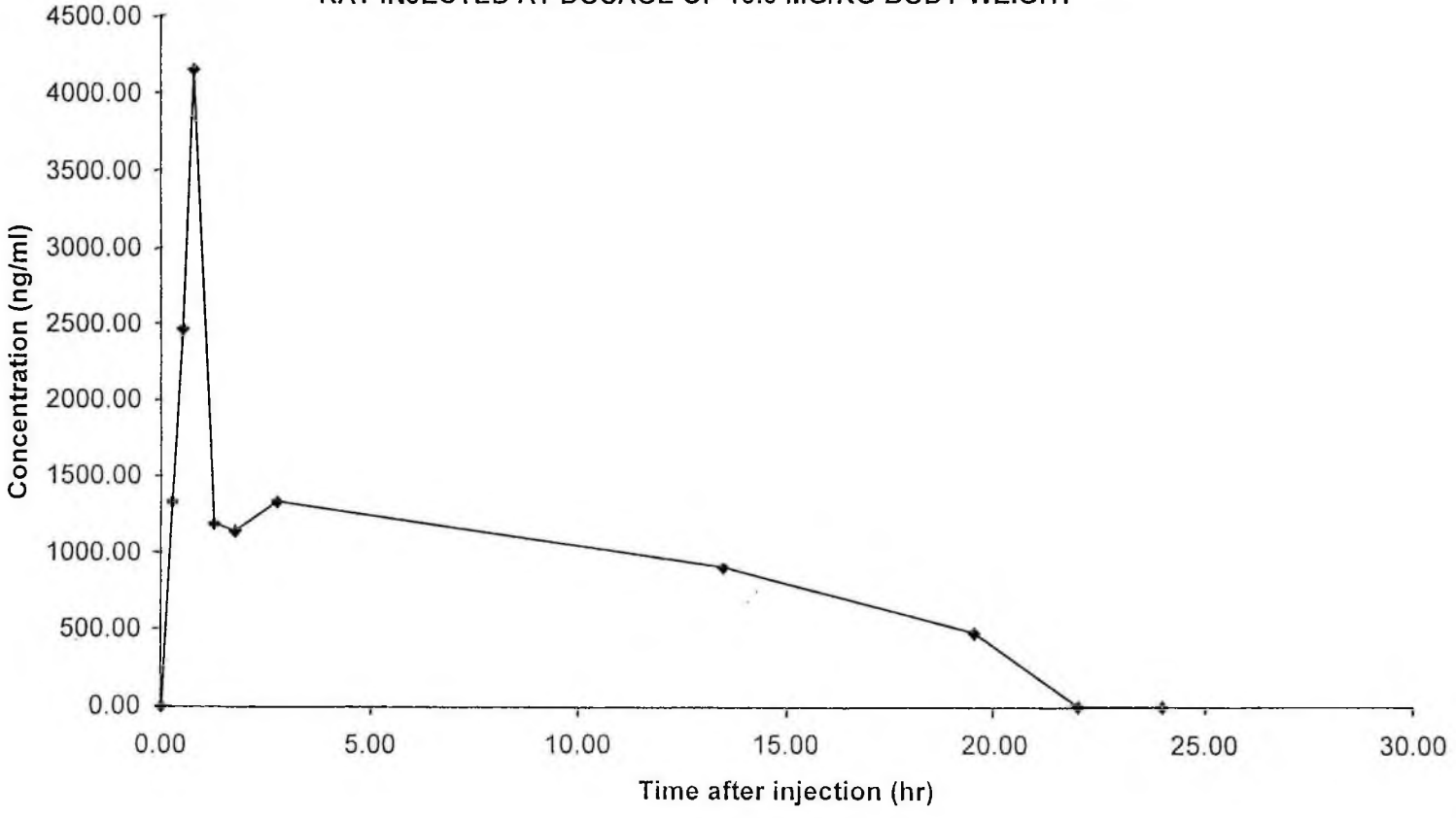
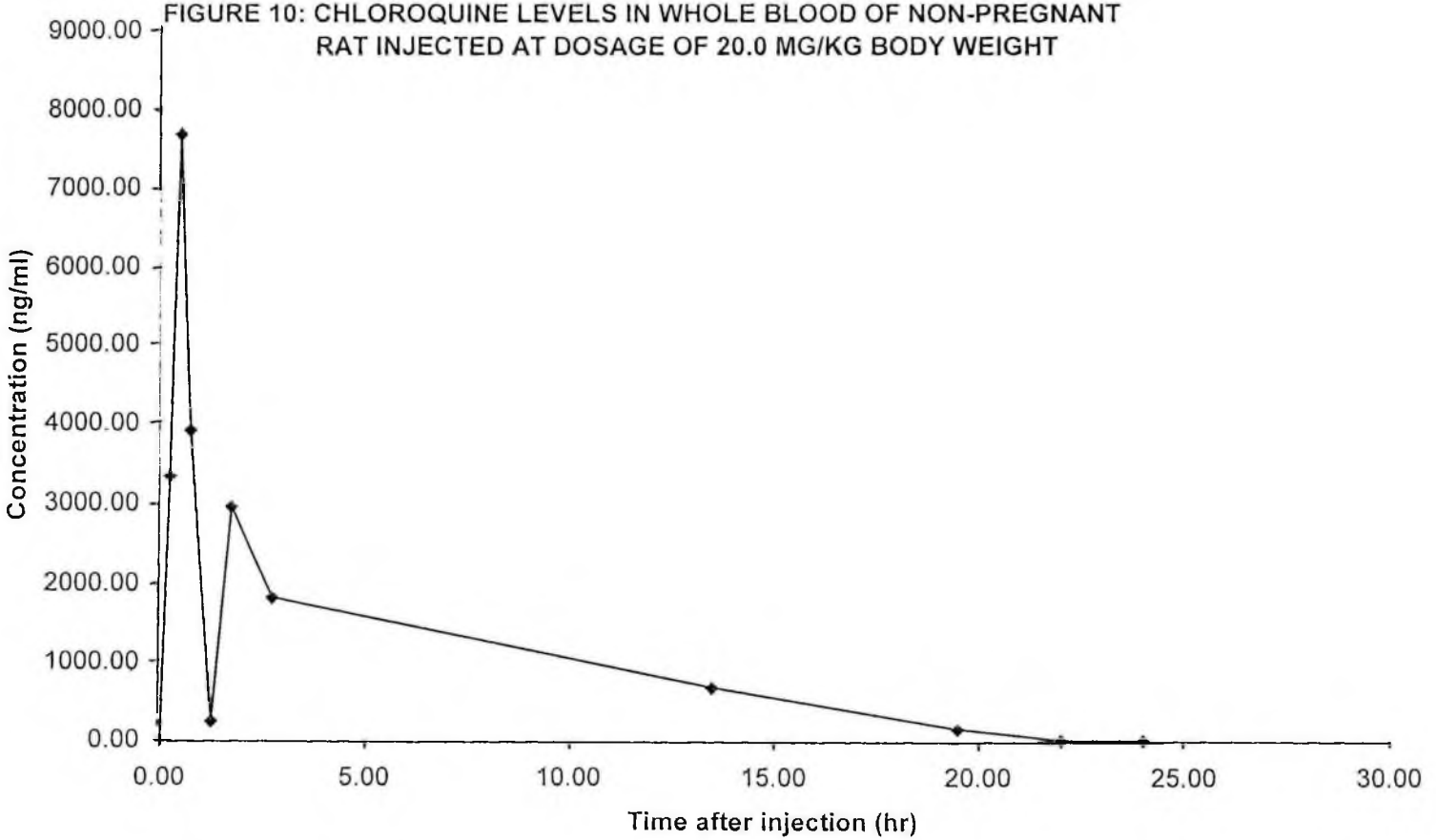


TABLE 6: CHLOROQUINE LEVELS IN WHOLE BLOOD OF NON-PREGNANT RAT INJECTED AT A DOSAGE OF 20.0 MG/KG BODY WEIGHT

Time(hr)	Peak Height Ratio (PHR)		Mean PHR	Conc (ng)		Conc (µM)
	A	B		Per 70µl	Per ml	
0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.74	0.66	0.70	233.30	3333.30	6.60
0.50	1.68	1.54	1.61	536.70	7674.90	14.90
0.75	0.78	0.85	0.82	273.00	3908.70	7.70
1.25	0.45	0.55	0.50	166.70	238.30	0.50
1.75	0.56	0.67	0.62	206.70	2955.30	5.80
2.75	0.42	0.34	0.38	126.70	1811.30	3.60
13.50	0.12	0.15	0.14	46.70	667.30	1.30
19.50	0.05	0.02	0.03	10.00	143.00	0.30
.22.00	0.00	0.00	0.00	0.00	0.00	0.00
24.00	0.00	0.00	0.00	0.00	0.00	0.00

FIGURE 10: CHLOROQUINE LEVELS IN WHOLE BLOOD OF NON-PREGNANT RAT INJECTED AT DOSAGE OF 20.0 MG/KG BODY WEIGHT



ng/ml at 19.50 hr after which no chloroquine was detected in the blood samples (see Tables 6 and 7; and Figure 10).

3.1.1.6 SUMMARY OF PHARMACOKINETIC STUDIES ON NON-PREGNANT FEMALE RATS

In summary, the five dosage levels of chloroquine diphosphate (7.5, 10.0, 12.5, 15.0 and 20.0 mg/kg body weights) produced different peak concentrations at different times in the blood of the female Sprague-Dawley rats. Whereas the average peak concentrations showed a decreasing trend in response to decreasing dosages, the times at which these concentrations were recorded were found to have no relationship with the dosages administered. It was also observed that no trace of the drug was detected in the blood samples beyond 13.50 hr for the treatment groups injected at doses of 7.5 mg/kg and 10.0 mg/kg body weight. Similarly, blood samples from rats injected at doses of 12.5 mg/kg body weight, 15.0 mg/kg body weight and the 20.0 mg/kg body weight showed no trace of drug in them after 19.50 hr. (See Tables 2-7; and Figures 6-10).

3.1.2 PREGNANT RATS

Analyses of blood samples taken from pregnant rats injected with chloroquine diphosphate showed no traces of the drug. The injection was done on day $9^1/_2$ of gestation using dosages of 0.0 mg/kg (as the control),

TABLE 7: AVERAGE CHLOROQUINE PEAK VALUES IN THE BLOOD OF NON-PREGNANT RATS

DOSAGE (MG/KG BODY WEIGHT)	PEAK VALUE		TIME AFTER INJECTION (HR)
	NG / ML	μ M	
7.5	1,125.54	2.50	1.75
10.0	1,688.31	3.30	0.25
12.5	1,811.80	3.50	1.25
15.0	4,157.00	8.10	0.75
20.0	7,674.90	14.90	0.50

7.5 mg/kg, 10.0 mg/kg and 12.5 mg/kg body weights.

The blood samples were taken approximately forty-eight hours later and just before the animals were sacrificed. The samples were not taken over the same period of time as was done for the non-pregnant ones because of the possibility of inflicting various stresses on the rats that might have adversely affected the animals hence the results or even induced abortions. This was primarily due to the rather stressful method of the squeezing blood from the coccygeal vein.

3.2 EMBRYOTOXICITY STUDIES

3.2.1 MORPHOLOGICAL ASSESSMENT AND SCORING

3.2.1.1 CONTROL

Eight female rats were used for this group with a litter size ranging from 5 to 6. In all, 42 embryos were examined. It was observed that the diameters of the visceral yolk sacs (YSD) ranged from 3.2 mm to 5.3 mm with a mean value of 4.1±0.6 mm (see Table 9); and Appendix C). The entire vasculature of the embryos was that of full yolk sac plexuses of vessels. The circulations were also vigorous with uniformly pink red blood cells.

The allantoies were generally found to have fused with the chorions. All but four of the embryos developed a curvature such that they described

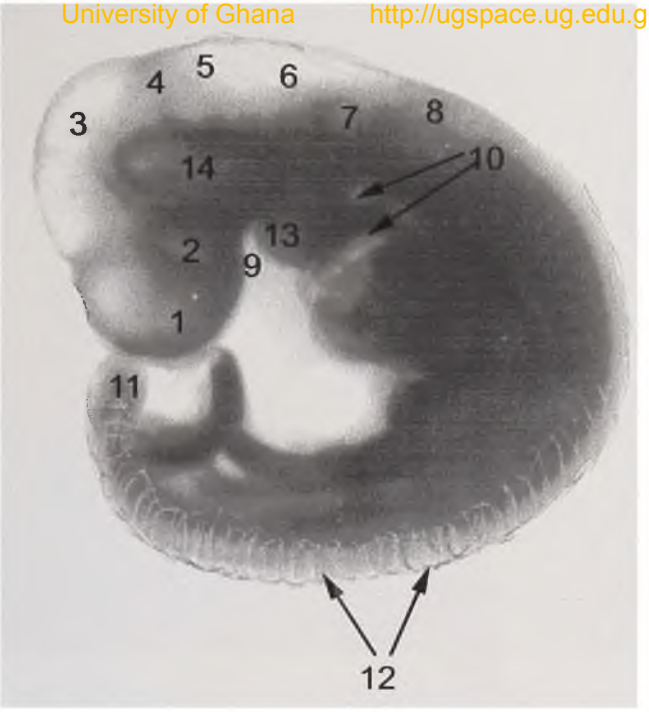


FIGURE 11:

An 11½ day-old embryo of the control group (x30.5).

LEGEND

1. Prosencephalon
2. Eye
3. Mesencephalon
4. Isthmus
5. Metencephalon
6. Myelencephalon
7. Auditory vesicle
8. Spinal cord
9. Stomodeum
10. Pharyngeal grooves
11. Tail
12. Somites
13. Mandibular process of first pharyngeal arch
14. Infundibulum

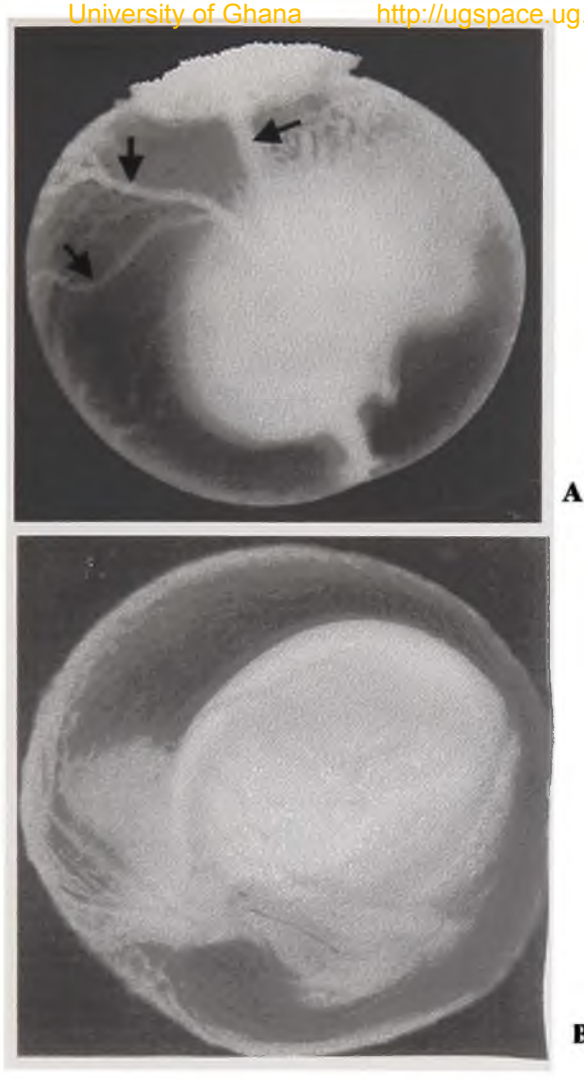
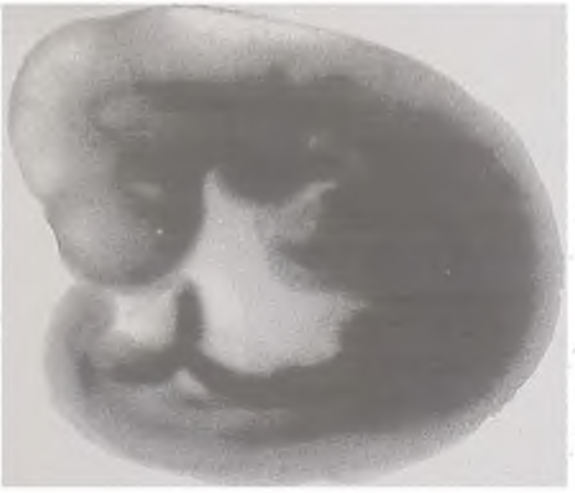


FIGURE 12:

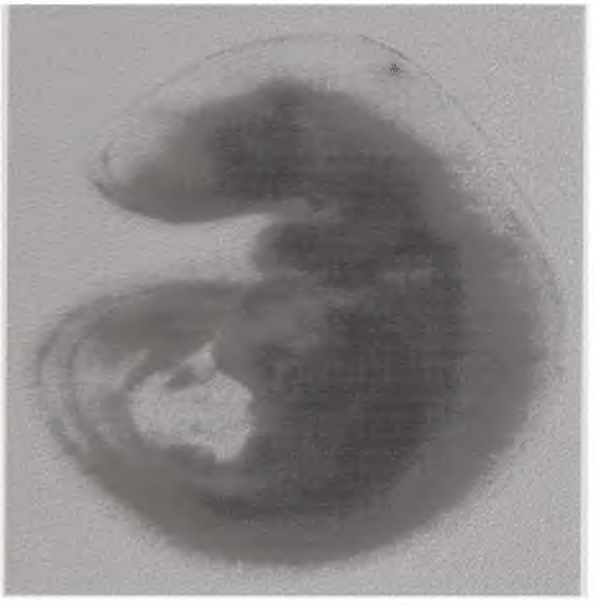
Embryos of the control group in their respective yolk sacs (x20.5).

A: Note the dorsally convex flexion of the embryo. Note also the full yolk sac plexus of vessels (arrows).

B: Note the uniformly vascularised yolk sac.



A



B

FIGURE 13:

Whole embryos of the control group with the yolk sacs removed (x25.0).

more than a complete circle with their tail buds consequently lying dorsal to their fore brains (see Figures 11 and 12). The embryos also showed a dorsal convexity and spiral torsion and had convoluted cardiac tubes.

The crown-rump lengths of the embryos in this group had a mean value of 3.0±0.6 mm ranging from a 1.9 mm to 4.3 mm. The mean head length (HdL) was 2.2±0.5 mm and ranged from 1.0 mm to 3.7 mm. The caudal neural tubes in this control group showed that almost 93% of the embryos had closed posterior neural pores. Also, their hindbrains exhibited pronounced pontine flexures with transparent roofs of the fourth ventricles (see Figures 11, 13 and 14).

Their midbrains were completely fused and the forebrains had developed visible telencephalic evaginations. The otocysts were present and had dorsal recesses while the optic systems had developed to the indented lens plate stage. The olfactory systems had distinct olfactory ridges. The pharyngeal bars I, II, and III were all present with the maxillary processes well demarcated by visible clefts anterior to bar I. At this stage, there was the formation of mandibular processes by the fusion of the first pharyngeal bars in the embryos (see Figure 11).

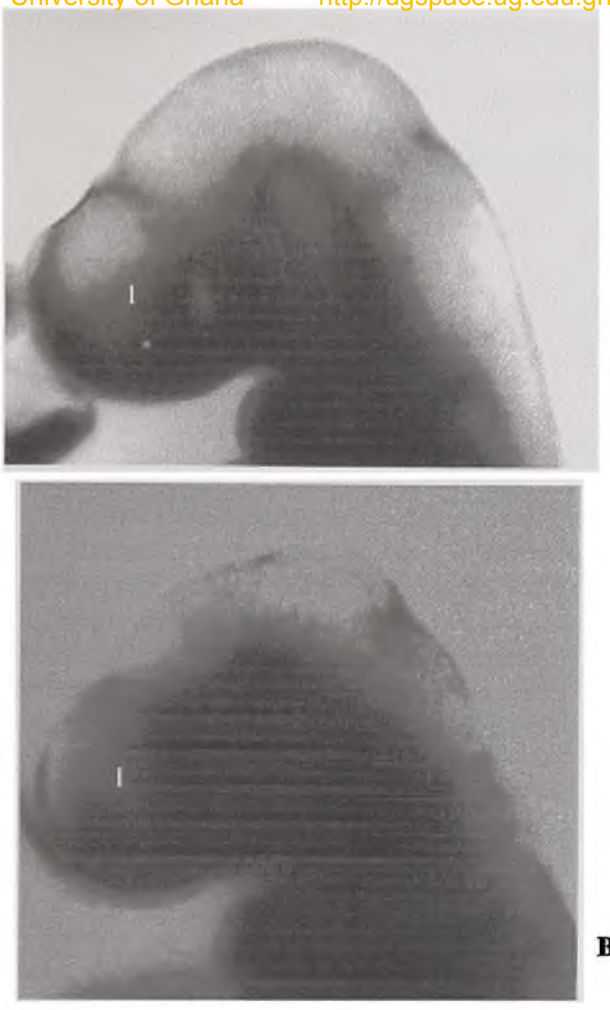


FIGURE 14 :

The head regions of embryos of the control (x32.5). Note the visible telencephalic evaginations (1) in both A and B. Refer also to Figure 11.

The forelimb and hindlimb buds had developed in all the embryos. The number of somites ranged from 27.0 to 36.0 with a mean of 31.7±2.9. Individual morphological scores ranged from 21.0 to 35.0 with a mean value of 29.1±3.8 (Table 9; and Appendix C).

In all, it was found that a total of four embryos, out of the total of forty-two examined (about 7%) possessed one or more abnormalities. These were abnormal axial rotation (one embryo), open anterior neuropore and hypoplasia of cranial neural tube (four embryos), microophthalmia (three embryos) and otic hypoplasia (two embryos). Based on these criteria, they were considered abnormal (see Table 8). The summary of the result is presented in Tables 8 and 9; Appendix C).

3.2.1.2 INJECTION OF CHLOROQUINE AT DOSAGE OF 7.5 MG/KG/BODY WEIGHT

For this treatment group, thirty-seven embryos were obtained from six parent rats with litter size ranging from 5 to 7 embryos. Almost 89% of embryos developed full yolk sac plexus of vessels. In the remaining embryos only the vitelline vessels were prominent. The yolk sac diameter measured from 1.8 mm to 6.1 mm with a mean of 4.2±0.9 mm. The red blood cells of majority of the yolk sacs showed relatively uniform pinkish appearance with vigorous circulation. The embryos were dorsally convex and had developed convoluted cardiac tubes.

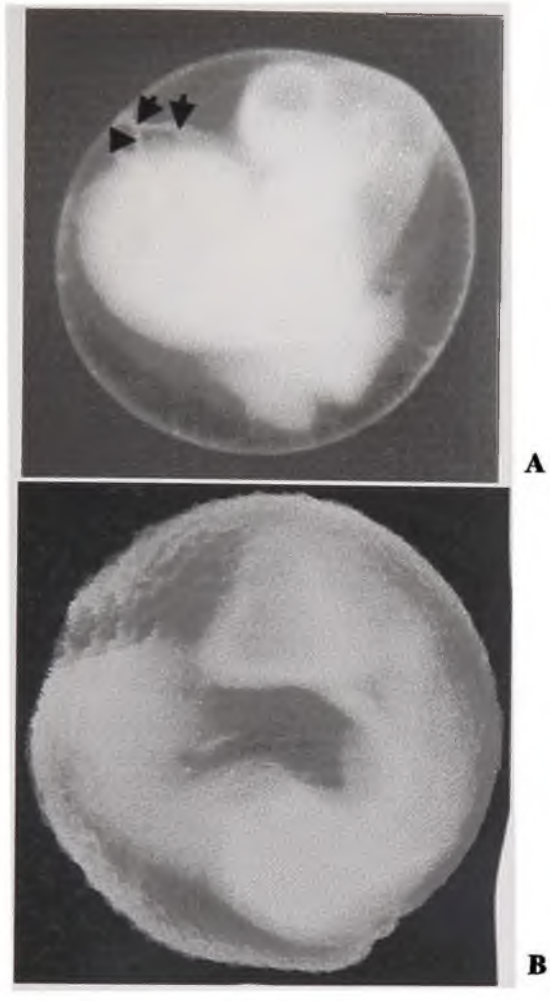


FIGURE 15:

Embryos of the 7.5 mg/kg body weight treatment group enclosed in their individual yolk sacs (x20.5). **A:** Relatively uniformly vascularised yolk sacs with the plexus of vessels (arrows). Note also the dorsally convex nature of the enclosed embryos in both A and B.

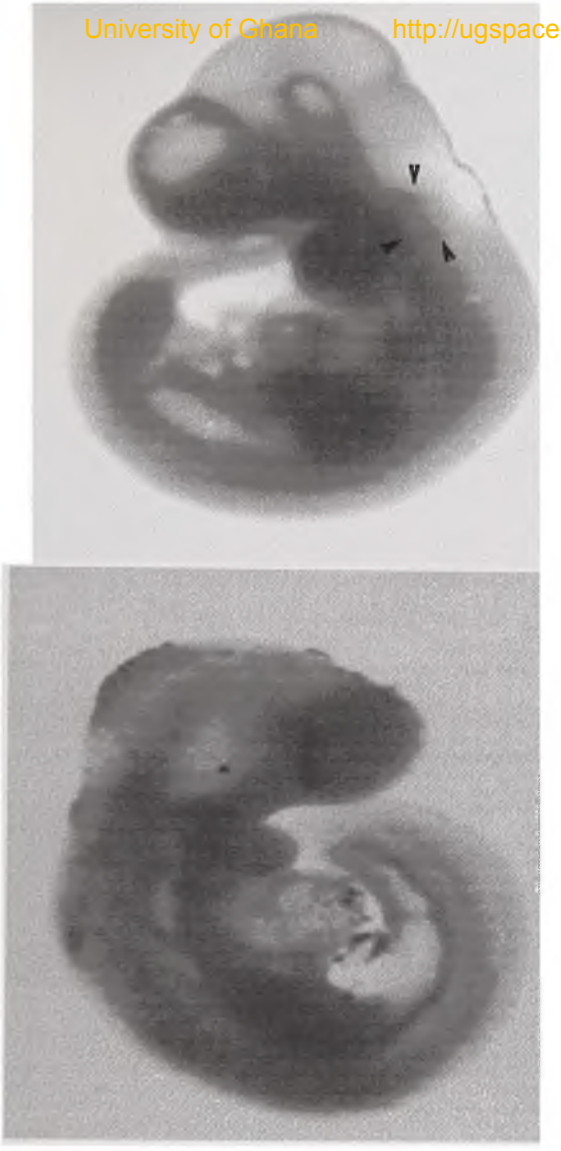
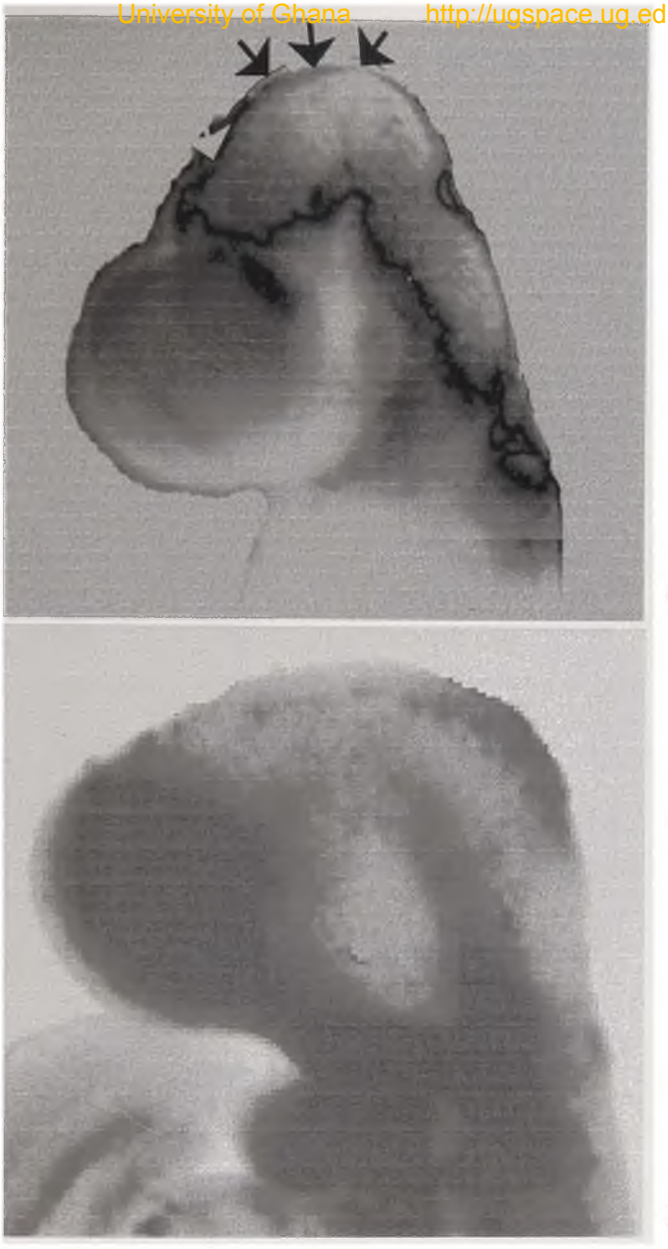


FIGURE 16:

Whole embryos of the 7.5 mg/kg body weight treatment group outside the yolk sacs (x25.0). **A:** Embryo with normally developed otic primordium (arrowheads). **B:** A generally dysmorphic embryo.



A

B

FIGURE 17:
Head regions of embryos of the 7.5 mg/kg body weight treatment group (x32.5).

A: Embryo showing closed cranial neural tube (arrows).

B: Dysmorphic- looking embryo.

It was also observed that six (16.2%) of the embryos had open anterior and posterior neuropores although the forebrains had developed visible telencephalic evaginations. The otic systems consisted of otocysts with dorsal recesses in about 93% of the embryos but the rest showed otocysts only. The optic systems generally had primary optic vesicles the olfactory systems had developed distinct olfactory ridges (see Figures 16 and 17). Pharyngeal bars I, II and III were visible with maxillary processes clearly demarcated by clefts anterior to bar I.

The crown-rump lengths of this group ranged from 1.2 mm to 4.8 mm with a mean value of 3.1±0.8 mm. The mean head length was 1.8±0.4 mm and ranged from 1.0 mm to 2.6 mm. The somites were generally flattened especially towards the tail end. The mean number was 32.2±3.1 with a range of 26.0 to 39.0. The total morphological scores were between 17.0 and 35.0 with a mean of 28.5±4.4.

Out of the 37 embryos, six (16.2%) were considered abnormal and showed characteristics such as abnormal axial rotation (one embryo), open anterior neuropore and hypoplasia of cranial neural tubes (five embryos), microophthalmia (four embryos), and otic hypoplasia (four embryos). (Table 8; and Figs 11, 16, 17 and Appendix C).

3.2.1.3 INJECTION OF CHLOROQUINE AT DOSAGE OF 10.0 MG/KG BODY WEIGHT

Forty-three embryos were used for this treatment. These were harvested from eight pregnant rats with litter size ranging from 6 to 7 per rat. The mean visceral yolk sac diameter in this group was 3.4±0.7 mm, with a minimum of 2.4 mm and a maximum of 4.9 mm. In general, the circulatory systems of the yolk sacs consisted of a full yolk sac plexus of vessels with about 10% of the conceptuses showing vitelline vessels and a few yolk sac vessels (see Figure 18). The red blood cells of the majority of the conceptuses were pale and the circulations were sluggish. The hearts also showed convoluted tubes. The crown-rump lengths ranged from 1.2 mm to 3.6 mm and a mean of 2.4±0.6 mm with mean head length of 1.5±0.4 mm. The majority of the embryos were dorsally convex.

About 12% of the embryos had open anterior neuropores and had developed mesencephalic brain folds at this stage. They also had visible telencephalic evaginations (see Figures 11, 19 and 20).

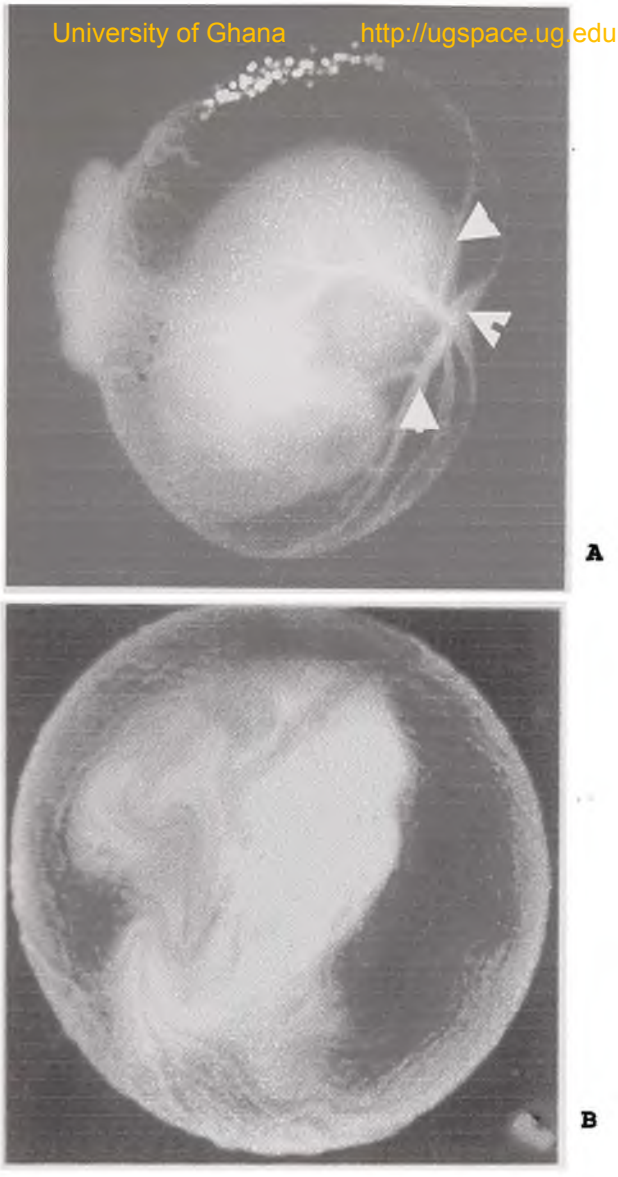


FIGURE 18:

Embryos of the 10.0 mg/kg body weight treatment group in their yolk sacs (x20.5). **A:** Note the vitelline vessels (arrowheads) with the few scattered yolk sac vessels. **B:** A uniformly vascularised yolk sac.

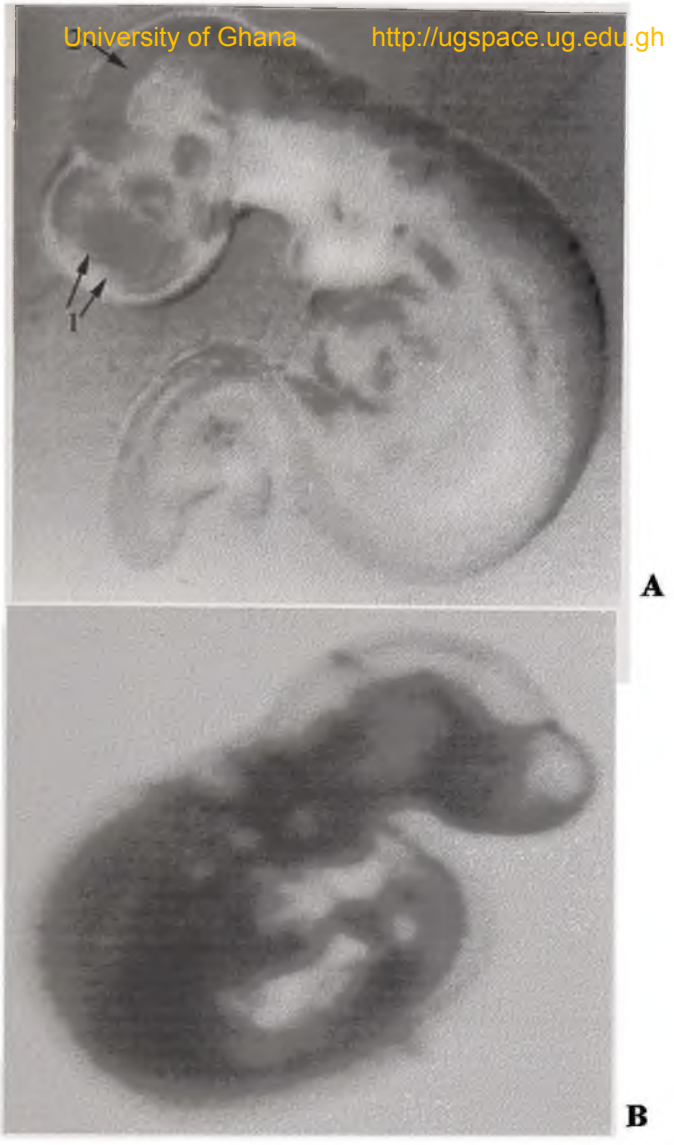


FIGURE 19:

Embryos of the 10.0 mg/kg body weight treatment group with their yolk sacs removed (x32.5). **A:** Note the the visible telencephalic evaginations (1) and the completely fused mesencephalon (2).

B: Observe the completely fused mesencephalic folds.

The otic systems were in the form of otic pits or otocysts. Most of the optic systems were with optic sulci and elongated optic premordia with the rest showing primary optic vesicles with optic stalk. The olfactory systems had generally developed to the olfactory plate stage. The pharyngeal bars I, II and III were all visible. Bar I showed signs of mandibular developments.

The forelimb buds were present while those of the hindlimbs were only rudimentary. The mean number of somites was 31.2±0.2.4 mm but those of individual embryos ranged from 26.0 to 36.0. These somites were flattened and tended to overlap one another making counting difficult. The total morphological scores for this treatment group were 25.0, 31.0 and 28.3±1.4 for the minimum, maximum and mean scores respectively (Table 9 and Appendix C).

In all, six of 43 embryos (about 7%) in this treatment group possessed characteristics such as abnormal axial rotation (three embryos), open anterior neuropore and hypoplasia of cranial neural tube (six embryos), microophthalmia (six embryos) and otic hypoplasia (four embryos), hence were considered abnormal (see Tables 8 and 9).

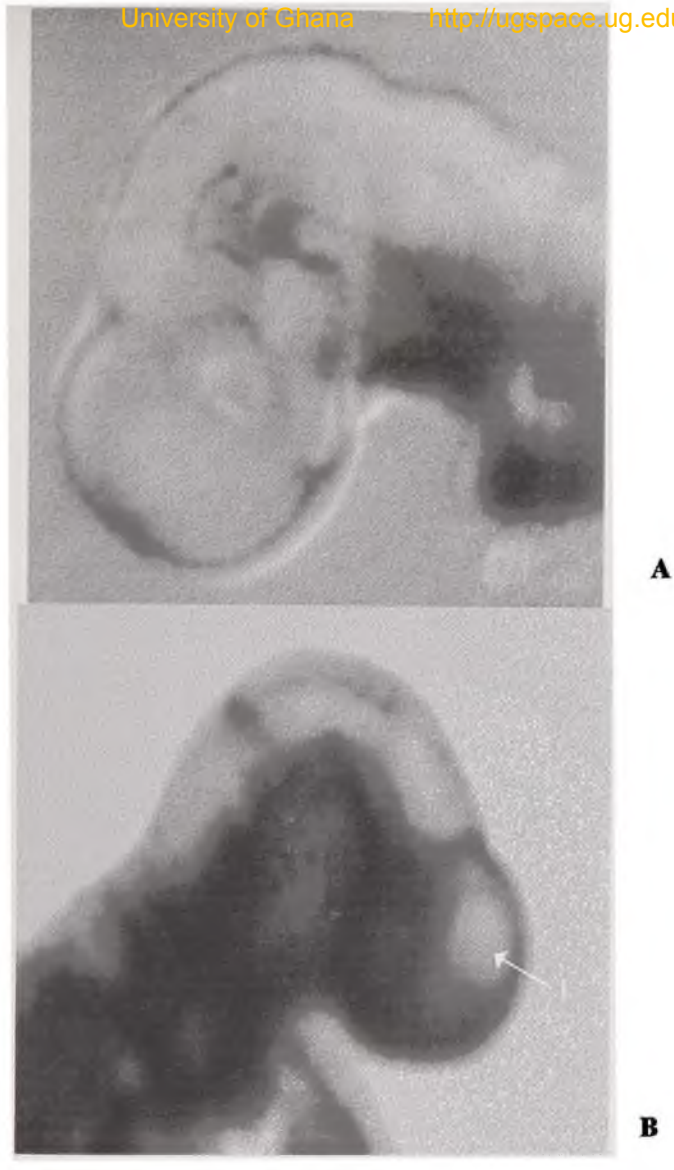


FIGURE 20:

Head regions of embryos of the 10.0 mg/kg body weight treatment group (x32.5). **A:** Normally - developed embryo. **B:** Note the visible telencephalic evaginations (1) .

3.2.1.4 INJECTION OF CHLOROQUINE AT DOSAGE OF 12.5 MG/KG BODY WEIGHT

Seven pregnant rats were used for this group. The litter size ranged from 6 to 7. A total of forty-one embryos were examined for this treatment group. Almost all embryos studied had soft and fragile visceral yolk sacs making the removal of the yolk sac difficult. It was also observed that about 16% of embryos had their visceral yolk sac systems made of vitelline vessels with few yolk sac plexus of vessels. The rest, however, developed full yolk sac plexus of vessels. The circulatory systems of the yolk sac showed sluggish circulation coupled with relatively pale red blood cells (see Figure 19). The diameters of the yolk sacs ranged between 1.8 mm and 5.3 mm and a mean of 3.0±0.9 mm (see Table 9 and Appendix C). The hearts mainly showed convoluted tubes.

Although the embryos showed dorsal convexity, the tail ends hardly touched the head regions. The embryos had open anterior and posterior neuropores and the forebrains showed completely fused prosencephalon. The otic systems showed combinations of otocysts, otic pits and a few with otocysts with dorsal recesses. The optic systems generally showed optic sulci in the majority of cases (Figure 23).

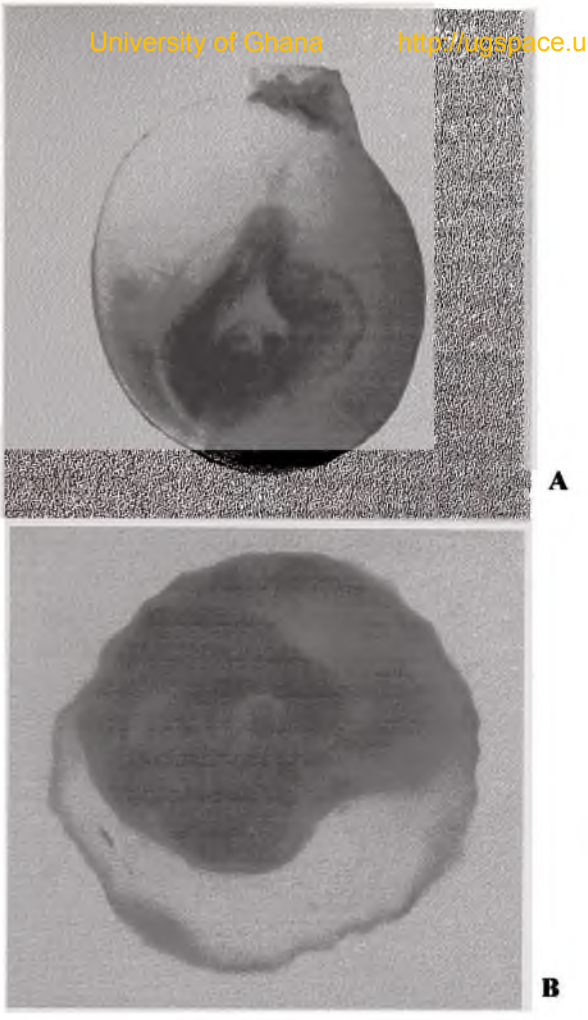


FIGURE 21:

Embryos of the 12.5 mg/kg treatment group in their yolk sacs (x20.5).

A: A normal embryo surrounded by a pale yolk sac.

B: A dysmorphic-looking embryo. Note the flaccid-looking yolk sac.

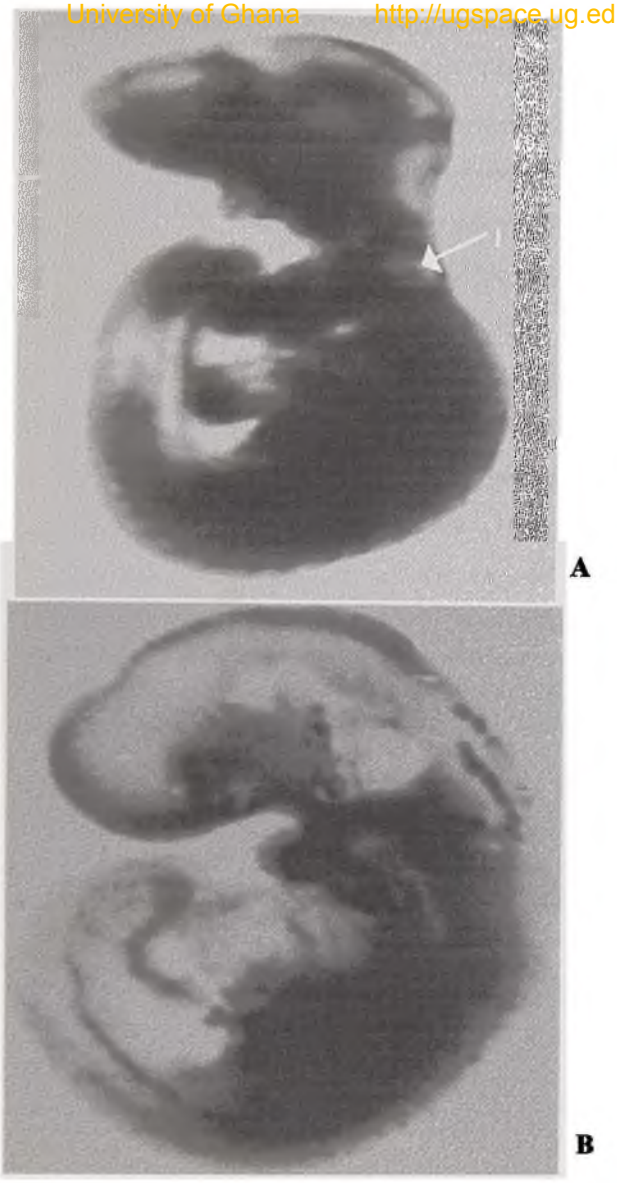


FIGURE 22:

Whole embryos of the 12.5 mg/kg body weight treatment group with their yolk sacs removed (x 32.5). **A:** Note the well developed otic primordium(1). **B:** A generally dysmorphic-looking embryo.

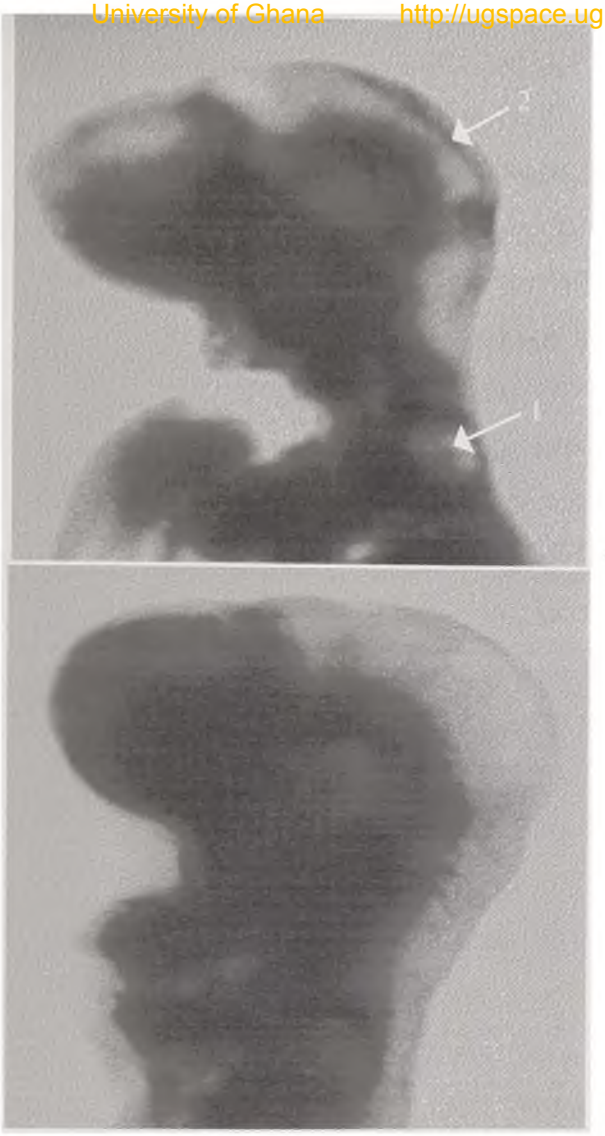


FIGURE 23:

Head regions of embryos of the 12.5 mg/kgbody weight treatment group (x32.5) **A:** Note the abnormally elongated head shape , the normally developed otic primordium (1) and the closed cranial neural tube (2).

B: Abnormal-looking head region.

The pharyngeal bars I, II and III were all visible with the maxillary processes well demarcated by visible clefts anterior to bar I. The mean crown-rump length was 2.0±0.9 mm with a range of between 1.1 mm and 4.5 mm. The forelimb buds were evident together with signs of hindlimb buds. The number of the somehow flat and overlapping somites were within the range of 28.0 to 35.0 and a mean value of 30.8±2.5. The total morphological scores also were between 24.0 and 33.0 with a mean of 28.7±2.4 (see Tables 8, 9; and Appendix C).

Seven out of the 41 (17%) embryos were found to be abnormal because they possessed either one or a combination of the following characteristics: abnormal axial rotation (one embryo), open anterior neuropore and hypoplasia of cranial neural tube (five embryos), microophthalmia (four embryos) and otic hypoplasia (three embryos). (See Figures 21, 22, 23; and Tables 8 and 9 for the summarised results).

3.3 DETERMINATION OF TOTAL PROTEIN CONTENT OF EMBRYOS

The details and a summary of the results for the determination of the total protein content of the embryos obtained are presented in Appendix C and Table 9 respectively.

3.3.1 CONTROL

The mean total protein content determined for this treatment group was 389.4±150.2 µg and ranged from 216.2 µg to 907.2 µg.

3.3.2 INJECTION OF CHLOROQUINE AT DOSAGE OF 7.5 MG/KGBODY WEIGHT

The total protein content obtained for individuals in this treatment group ranged from 155.6 µg to 813.0 µg with a mean of 380.2±173.5 µg.

3.3.3 INJECTION OF CHLOROQUINE AT DOSAGE OF 10.0 MG/KG BODY WEIGHT

The mean total protein content of this group was 208.8±82.0 µg and ranged from 80.8 µg to 390.6µg.

3.3.4 INJECTION OF CHLOROQUINE AT DOSAGE OF 12.5 MG/KG BODY WEIGHT

The total protein content of individual embryos in the group ranged from 56.6 µg to 695.1 µg with a mean of 174.3±160.3 µg.

TABLE 8: SUMMARY OF MORPHOLOGICAL ABNORMALITIES

Treatment/Dosage (Mg/kg bd. Wt.)	Total Number Abnormal	Axial Rotation	Neural Tube	Otic Primordium	Optic Primordium
0.0 n = 42	4 (10%)	1 (2%)	1 (10%)	2 (5%)	3 (7%)
7.5 n = 37	6 (16%)	1 (3%)	1 (14%)	4 (11%)	4 (11%)
10.0 n = 43	6 (14%)	3 (7%)	6 (14%)	4 (9%)	6 (14%)
12.5 n = 41	7 (17%)	1 (2%)	5 (12%)	3 (17 %)	4 (4%)

TABLE 9: MORPHOLOGICAL ASSESSMENT AND TOTAL PROTEIN CONTENT (MEAN ± SEM)

Treatment (Dosage) in Mg/kg Body Wt.	YSD (mm)	Total Protein Content (µg)	Crown-Rump Length (mm)	Head Length (mm)	Number Of Somites	Morphological score
0.0 n = 43	4.1 ± 0.6	389.4± 150.2	3.0 ± 0.6	2.2 ± 0.5	31.7 ± 2.9	29.1± 3.8
7.5 n = 37	4.2± 0.9	380.2 ± 173.5	3.1± 0.8	1.8 ± 0.4*	32.2 ± 3.1	28.5± 4.4
10.0 n = 43	3.4± 0.7*	208.8± 82.0*	2.4±0.6*	1.5 ± 0.4*	31.2 ± 2.4	28.3± 1.4
12.5 n = 41	3.0± 0.9*	174.3±160.3*	2.0±0.8*	1.4 ± 1.3*	30.8 ± 2.5	28.7± 2.4

* Significant difference from controls at $P < 0.001$, by the Bonferroni Multiple Comparison Test.

CHAPTER 4

DISCUSSION

4.1 PHARMACOKINETIC STUDIES

4.1.1 NON-PREGNANT FEMALE RATS

The study indicated that chloroquine diphosphate injected into the non-pregnant female Sprague-Dawley rats was rapidly absorbed. The five dosage levels of the chloroquine diphosphate produced various peak concentrations in the blood of the rats. Peak whole blood concentrations occurred between 0.25 hr and 1.75 hr after the introduction of the drug. It was also observed that the higher the dosage level the higher the peak blood concentration and the earlier the peak time (Table 7).

4.1.1.1 RELATIONSHIP BETWEEN THE DOSAGE OF CHLOROQUINE AND PEAK BLOOD CONCENTRATION

Bergqvist and Domeij-Nyberg (1983) have reported that the higher the dosage of chloroquine administered, the higher the peak blood concentration. Their results also showed that this relationship exists irrespective of the route of administration of the drug. They further indicated, however, that there is no direct quantitative conversion factor between the dosage and the corresponding blood levels of the drug.

With the findings of Gustafsson *et al.* (1983), Adjepon-Yamoah *et al.* (1986) and Bergqvist and Domeij-Nyberg (1983), together with the earlier estimation by Frisk-Holmberg *et al.* (1979), a cautious attempt may be made to establish a relationship between the dosages administered and the peak concentrations of chloroquine in the blood of the non-pregnant female rats obtained in the present study. Further attempts may also be made to establish a relationship between the measured peak concentrations of chloroquine in the blood of the non-pregnant female rats and the concentration of the culture medium applied by Tagoe and Ofori-Adjei (1995).

According to Frisk-Holmberg *et al.* (1979), the whole blood concentrations of chloroquine were about 8 to 10 times higher than those obtained in plasma and serum. This estimation was arrived at after they administered a series of dosages of 125 mg of chloroquine phosphate, both orally and parenterally, to humans. They finally established such a quantitative relationship based on the peak whole blood concentrations (ranging from 332 ng/ml to 759 ng/ml) and plasma concentrations (ranging from 32 ng/ml to 89 ng/ml) of the drug measured in the subjects.

Gustafsson *et al.* (1983) on the other hand, gave a single oral dose of 300 mg of chloroquine phosphate to adult humans and measured plasma concentrations ranging from 94 ng/ml to 120 ng/ml.

In a similar study on humans by Adjepon-Yamoah *et al.* (1986), a single oral dose of 620 mg chloroquine base (10-12 mg/kg body weight) was administered (almost double that applied by Gustafsson *et al.* (1983)). The peak concentration of the drug in the whole blood of subjects ranged from 1,058 ng/ml to 1,113 ng/ml. Adjepon-Yamoah *et al.* (1986) made reference to the results of Gustafsson *et al.* (1983) to justify their findings. This was because the peak concentrations in plasma (94 ng/ml to 120 ng/ml) were about 8 to 10 times lower than those measured in whole blood (1,058 ng/ml to 1,113 ng/ml).

The above comparison between Adjepon-Yamoah *et al.* (1986) and Gustafsson *et al.* (1983) vis-à-vis the findings of Frisk-Holmberg *et al.* (1979) may also apply to the peak concentration of chloroquine determined in the whole blood of the rats in the present experiment as well as the concentration of chloroquine applied by Tagoe and Ofori-Adjei (1995) in their *in vitro* study. These comparisons, however, are being done against differences in subjects (human versus rats), routes of drug administration (oral, intraperitoneal and direct application) and the mode of experimentation (*in vivo* versus *in vitro*).

Therefore, when these differences are accounted for, the peak whole blood concentrations of chloroquine obtained in the present may be said to be similar to the 500 ng/ml (approximately 0.97 μ M) administered by Tagoe

and Ofori-Adjei (1995) in their *in vitro* study of rat embryos (see Table 7). This serum concentration, Tagoe and Ofori-Adjei (1995) indicated, was in the range of the serum level achievable after the long-term use of the drug for malaria treatment (see Mackenzie, 1983a).

4.1.1.2 RELATIONSHIP BETWEEN DOSAGE OF CHLOROQUINE AND THE TIME OF PEAK WHOLE BLOOD CONCENTRATION IN NON-PREGNANT RATS

Although the times recorded for the peak concentrations of the drug in the blood of the non-pregnant rats generally bear no direct quantitative relationship with the dosages administered, certain seeming contributory factors to the observed trend in this situation are worth noting. The physiological and environmental factors are some of the immediate possible sources that can be cited as being responsible for the observed trend (see Figures 6-10).

The clotting of blood in the course of taking samples into the capillary tubes may be cited as a possible factor too. This situation as expected, had caused the release of more of the drug from the platelets and erythrocytes during the process of a possible haemolysis. This occurrence was precipitated in part by the mode of collection of the blood samples, which tended to inflict some amount of pain stress on the rats. This observation demonstrated the peculiar and interesting pharmacokinetic behaviour of

chloroquine, that chloroquine accumulates in platelets and erythrocytes, hence reaching a concentration of approximately a four hundred fold in erythrocytes than in serum (Solomon *et al.*, 1967).

From the foregoing discussion, it may be deduced that a cautious comparison can therefore be made between the results obtained in peak serum and whole blood chloroquine concentrations. This comparison, however, is being done against an earlier assertion by Domeij-Nyberg *et al.* (1983) that a universal conversion of serum concentrations to whole blood concentrations by means of a conversion factor is not possible as ratios between serum and whole blood concentrations vary between individuals.

Further, from the explanations by Domeij-Nyberg *et al.* (1983), it is suggestive that the determination of whole blood concentrations of chloroquine, indeed, has some advantages compared with serum and plasma concentrations. This is because the drug is found to concentrate in formed elements with blood platelets cited as a major source of erroneous measurement of plasma and serum levels of the drug (Adjepon-Yamoah *et al.*, 1986).

It was in this light that the present investigation, as a matter of priority and pharmacological expediency, determined the chloroquine kinetics in

whole blood. This action will, at least, minimize the potential methodological pitfalls associated with the determination of the concentrations of the drug in plasma and serum.

4.1.2 PREGNANT RATS

The blood samples from this group of animals were taken at the time of sacrifice to avoid inflicting stresses on the animals that might have adversely affected the results or possibly induced abortions. This was primarily due to the rather stressful method of the collection of the blood samples by squeezing from the coccygeal vein as described earlier under item 2.1.5 of Chapter 2.

Analyses of blood samples taken from pregnant rats injected with chloroquine diphosphate on gestation day $9^1/_2$ showed no traces of the drug. This apparent absence of chloroquine from the samples may be explained on the basis some observations made by Gustafsson *et al.* (1983). They indicated that chloroquine is rapidly removed from the blood and then concentrated in organs such as the liver, spleen, lungs and blood cells as well as platelets thereby depleting the blood of the drug in no time.

4.2 EMBRYOTOXICITY STUDIES

4.2.1 PREAMBLE

Results obtained from the pharmacokinetic studies indicated that the three dosage levels, 7.5 mg/kg body weight, 10.0 mg/kg body weight and 12.5 mg/kg body weight produced peak whole blood concentrations of chloroquine comparable to those attainable during the long term use of the drug (See Mackenzie, 1983a). These dosages were, therefore, chosen for the embryotoxicity studies (see Tables 2-7; and Figures 6-10).

The three dosage regimens of chloroquine diphosphate administered intraperitoneally on the pregnant rats on gestation day $9^1/_2$ over the 24 hr period generally caused decreases in all the growth parameters as well as the total protein content of the embryos in this study. The dosages also caused various degrees of dysmorphogenesis in the embryos. The results in general agreed with those reported by Ambroso and Harris (1993) and Tagoe and Ofori-Adjei (1995). The results of the current investigation, however, showed less severe retardations as compared with Ambroso and Harris (1993) and Tagoe and Ofori-Adjei (1995) (see Tables 8 and 9; and Figures 11-23).

4.2.2 EFFECTS OF THE THREE DOSAGES OF CHLOROQUINE ON THE YOLK SAC OF EMBRYOS

On the growth parameters, it was realised that whereas the dosage of 7.5 mg/kg body weight reduced the yolk sac diameter of the embryos to 95% those of 10.0 mg/kg body weight and 12.5 mg/kg body weight caused reductions to 80% and 71% respectively. These statistically significant reductions particularly confirmed the results of Tagoe and Ofori-Adjei (1995). Other parameters observed included the underdevelopment of the vitelline vascular plexus (see Table 9 and Figures 12,15 and 18), sluggish circulation as well as relatively pale red blood cells of treated embryos as opposed to the vigorous circulation and the uniformly pink red blood cells of the controls.

It has been reported that the endodermal cells of the visceral yolk sac absorb various chemicals (William *et al.*, 1976) and may cause accumulation of high levels of these drugs in them (Ambroso and Harris, 1993). Therefore, the flaccid and tender yolk sacs of the drug-treated embryos may be due to the possible damaging effects of chloroquine on the structures during accumulation. As a result, substantial levels of the drug were possibly channeled to the embryos through the placenta (Wilson, 1973). The damage to the yolk sacs may have compromised their protective (Goldman, 1909; Wilson, 1951; Brent, 1971), absorptive (William *et al.*, 1976) and nutritional (Merker, 1970) functions.

The damage(s) caused to the embryos by the drug may be attributable, in part, to the fact that some functions of the visceral endodermal cells of the yolk sac are altered by several teratogens. The inhibition of absorption and transport of calcium, sodium and sulphate ions in the yolk sac of rat following trypan blue treatment have been reported (Kernis *et al.*, 1969). Similarly, the inhibition of the transport of the amino acid, valine, in the yolk sac after chlorambucil injection (Kernis, 1971). Also, the *in vitro* incorporation of both glucose and glycine into the rat yolk sac in the presence of chloramphenicol and puromycin have been reported to be compromised (Fridhandler and Zipper, 1964).

Adverse effects of the drug on the visceral yolk sac of the embryo may have had a bearing on the consequent growth retardations and dymorphogenesis observed in the present study since the visceral yolk sac plays a vital role during embryogenesis (Brunschwig, 1927). Goldman (1909) indicated that the real roles of the two placentas (the villiary highly vascularised yolk sac together with the discoidal chorioalloantoic placentas) during the critical period of organogenesis were not well understood. He, however, postulated that the role of the visceral yolk sac in the presence of teratogens was for protection. This was based on his observation that the dye, trypan blue, was extensively absorbed by the visceral yolk sac long before it was even known to be a teratogen. The suspected protective ability of the yolk sac was further enforced by the

observation that trypan blue was sequestered by the endodermal cells and, more importantly, that the teratogenic activity of the dye ceased about the same time that the visceral yolk sac completely encircled the embryo (Wilson, 1959).

It may therefore be speculated that the individual dosage regimens of chloroquine applied in the present study were, in part, responsible for the observed effects on the visceral yolk sacs of the drug-treated embryos. This may be due to the adverse effects of the accumulation of the drug on these structures thereby compromising their essential functions.

4.2.3 EFFECTS OF THE THREE DOSAGES OF CHLOROQUINE ON THE TOTAL PROTEIN CONTENT OF EMBRYOS

The total protein contents of the embryos of the drug-treated groups were lower when compared with the controls. The dosages of 7.5 mg/kg body weight had 98%, 10.0 mg/kg body weight, 53% and the 12.5 mg/kg body weight 45%. These dose-related decreases also confirm those observed by Ambroso and Harris (1993) in their study.

This observation may be explained on the basis of the mode of action of the drug, which is thought to intercalate between DNA base pairs (O'Brien *et al.*, 1966) and therefore interferes with DNA and protein syntheses (Ciak and Hahn, 1966). Again, it has been postulated that by its

accumulation in lysosomes, chloroquine delays or even disrupts lysosomal functions (Zvailer, 1964; Lie and Schofield, 1973) including the inhibition of DNA and RNA polymerase reactions (Cohen and Yielding, 1964) hence the syntheses of DNA and protein.

Another possibility was that chloroquine under the present circumstance may as well have caused the inhibition in lysosomal enzymatic activity and pinocytosis when accumulated in the visceral yolk sac cells. This inhibition consequently caused a reduction in the ability of the cells to digest proteins and related nutrients (Beck *et al.*, 1967; William *et al.*, 1976). The assertion by William *et al.* (1976) was supported by the observation that protein and other materials, such as iron, pass through the parietal wall of the yolk sac and then are absorbed by the endodermal cells of the yolk sac's visceral wall. These materials are further channeled to the embryo via the branches of the vitelline veins lying in the mesenchyme beneath the visceral endoderm (Anderson, 1959; Fedinec, 1962; Lambson, 1969).

The impaired digestive and transportation functions of the endodermal cells of the yolk sac therefore resulted possibly, in the failure of the movement of these nutrient materials, especially proteins, from the visceral yolk sac to the developing embryo hence the observed trend in this investigation. Earlier, according to Everett (1935), the villiary highly

vascularised yolk sac placenta in the rat together with the discoidal chorioalloantoic placenta, were found to be the organs that serve as the main media for maternal-embryo exchanges, hence substantiating the evidence assigning the animal two functional placentas during gestation.

Moreover, it has been suggested that the absorptive ability of the visceral yolk sac endoderm was inherent in the cells. This was because these cells continue to absorb the relevant substances even when transplanted to an *in vitro* site (Ferm *et al.*, 1960) and also possess the ultrastructural arrangement characteristic of protein-absorbing cells (Wislocki, 1955; Padykula *et al.*, 1966). This, therefore, means that protein can be absorbed by the visceral yolk sac cells, digested intracellularly and the end products subsequently released from the cells (Merker, 1970; Waddell, 1972; Williams *et al.*, 1976). This digestion process has therefore been suggested to be necessary for supplying the embryo with nutrients (Adcock *et al.*, 1973). These findings might explain the crucial role of the visceral yolk sac in the survival of the embryo in general.

Yolk sac hematopoiesis could also be affected to a considerable extent due to the adverse effects of chloroquine inhibition of the release of iron from transferin (Ambroso and Harris, 1993). It has therefore been suggested that damage caused to the yolk sac might eventually inhibit the transport of iron from mother to embryo. This inhibition consequently resulted in

the decrease in the production and functions of both the haemoglobin and erythrocytes in the embryos. These may have caused anoxia in the embryos thereby contributing to the growth retardations observed (Miki *et al.*, 1988).

With all these possibilities, one may only suggest that a combination of several factors was responsible for the observations made. Follow-up studies would be required to validate the claims advanced in these instances.

4.2.4 EFFECTS OF THE THREE DOSAGES OF CHLOROQUINE ON HEAD AND CROWN-RUMP LENGTHS OF EMBRYOS

The head length measurements of the drug-treated groups showed reductions when compared with the controls. The head length of the 7.5 mg/kg body weight treatment group measured 81% of the control, that of the 10.0 mg/kg body weight group, measured 67% of the control and the 12.5 mg/kg body weight group measured, 65% of the control. The crown-rump length of the embryos showed that the 7.5 mg/kg body weight treatment group measured 104% of the control; 10.0 mg/kg body weight treatment group measured 80% of the control and the 12.5 mg/kg body weight treatment group measured 70% of the control. Except for the crown-rump length for the 7.5 mg/kg body weight treatment group, all the

other treatment groups showed statistically significant differences compared with the controls at $P<0.05$ (see Table 9 and Appendix C).

In general, since the normal growth and development of organisms involve cell division and increases in protoplasm, any factors that tend to disrupt the normal cell division inevitably adversely affect the processes of growth and development. Therefore, it is possible a disruption of one or both of such vital processes in the embryos caused by the drug was responsible for the observed significant reductions in the crown-rump and head lengths in the present study.

4.2.5 EFFECTS OF THE THREE DOSAGES OF CHLOROQUINE ON NUMBER OF SOMITES AND MORPHOLOGICAL SCORES OF EMBRYOS

The number of somites as well as the total morphological score, which give an indication of the overall growth status of the embryos, also showed a decreasing trend as the concentration of the drug administered increased. For the number of somites, the following percentages (of controls) were measured for the respective treatment groups:-7.5 mg/kg body weight 102%, 10.0 mg/kg body weight, 98% and the 12.5 mg/kg body weight, 97%. The morphological scores expressed as percentages of the controls were: - 98% for the 7.5 mg/kg body weight, 97% for the 10.0 mg/kg body weight and 98% for the 12.5 mg/kg body weight (all not significant at $P<0.05$) (see Table 9 and Appendix C). The above-stated

factors that have been cited as contributory to the generally decreasing trends in the measured parameters could as well be applied here. This is primarily because these factors all had the potential of causing decreases in the quality of the relevant cells required for both replacement and repair. The above observations on the growth are similar to those of Tagoe and Ofori-Adjei (1995) in spite of the genetic differences in the animals used (Wistar versus Sprague-Dawley rats).

4.2.5 EFFECTS OF THE THREE DOSAGES OF CHLOROQUINE ON DYSMORPHOGENESIS IN THE EMBRYOS

Striking observations made during the morphological assessment were the dysmorphic features exhibited by the embryos (see Table 8; Figures 11, 13, 14, 16, 17, 19, 20, 22 and 23). These involved open anterior neuropore, hypoplasia of cranial neural tubes, abnormal axial rotation, microophthalmia and otic hypoplasia. These abnormalities, which were not peculiar to any specific treatment group, have been reportedly caused by several other teratogenic agents as well (Shepard, 1989).

The role of the chloroquine diphosphate applied in the dysmorphogenesis caused in the embryos in this present study may be explained partly by observations made by Beckman and Brent (1984). According to these authors, the initial response of the embryos and foetuses to teratogens in general, be it molecular, biochemical or biophysical, may result in

different sequences of cellular changes including cell death, faulty cellular interaction-induction, reduced biosyntheses of substrates, impaired morphogenetic movements and mechanical disruption. These processes they explained, eventually lead to final defects including developmental anomalies, embryonic and foetal growth retardation and even death. A combination of these processes possibly may account for the dysmorphogenesis observed in the drug-treated embryos.

The current research considered any embryo with any two or more of the above characteristics as abnormal. Accordingly, the drug-treated embryos showed abnormality rates of 16% for the 7.5 mg/kg body weight treatment group, 14% for the 10.0 mg/kg body weight treatment group and 17% for the 12.5 mg/kg body weight treatment group. The control had 10%. These values when compared using Fisher's test at $P<0.05$ were not significant though.

In humans, it is reported that about 3% of infants (30 per 1000 births) have an obvious major anomaly at birth, 6% at six years and 8% at five years (King *et al.*, 1965; Nelson and Holmes, 1989; Khoury, 1995). Therefore, the 10% rate of dysmorphogenesis recorded (4 out of the 42 embryos) for the control group may be considered a rather high rate. This may be partially explained by the fact that in *in vivo* experiments such as this, embryos, conceptuses and foetuses with extreme

deformities/abnormalities caused by factors other than those under investigation cannot be excluded prior to the application of the treatment unlike the *in vitro* experiments. This suggests that the dysmorphogenesis in the control was possibly initiated by other intrinsic and extrinsic factors like apoptosis, prior to the administration of the drug.

The observed rates for all the treatment groups may be as a result of the effect of the chloroquine diphosphate applied as well as other factors. Therefore, it may be suggested that the application of the drug enhanced the process of dysmorphogenesis in the embryos bringing about the dose-related responses observed. The 48-hr period of the exposure of the embryos to the drug in this instance may be viewed to be too short a time to allow for any meaningful healing process in the embryos. Accordingly, a longer period of experimentation will help explain the observation better than at present.

Another interesting contributory factor to the rather high rate of dysmorphogenesis in both the control and drug-treated embryos may be related to the phosphate-buffered saline used as the solvent in this experiment. As explained by Anderson and Morse (1966), a vehicle or solvent for the dissolution of a drug could alter the outcome of an experiment. This finding was based on an experiment they performed using different solvents for folic acid antagonists to elucidate varying rates

and forms of deformities in adult rats. Ferm (1966) also demonstrated that such vehicles and solvents may themselves be teratogenic to appreciable degrees.

It may therefore be speculated that the 10% rate of dysmorphogenesis in the control embryos may, partly, be attributable to the vehicle used as discussed above. This also implies that the effects of the phosphate-buffered saline and the chloroquine diphosphate applied together collectively contributed to the rates of dysmorphogenesis observed in the treatment groups.

To verify the role played by the phosphate-buffered saline in the dysmorphogenesis, it will be appropriate that two sets of control (one treated with phosphate-buffered saline and the other not) are carried out. This method will ensure a comparison between the natural or normal wastage rate and that caused by the phosphate-buffered saline in the animals.

On the statistically insignificant rates of dysmorphogenesis recorded in all the groups, it was also possible that the concentrations of the drug were so high that they stimulated microsomal enzymes in the systems of the embryos, although the chloroquine levels in the blood of the embryos and conceptuses were not determined. These enzymes according to King *et al.*

(1965) and Conney and Burns (1972), facilitate the auto-metabolism of drugs hence avoiding a possible build-up in both the parents and the embryos to cause any appreciable damage to the embryos. This observation could be a result of a related teratogenic experiment performed in which Conney and Burns (1972) administered a common dosage of the drug chlorcyclizine on two sets of pregnant rats for two different periods of time, 4 and 16 days respectively. Their results primarily showed that the group on which the drug was administered over a 4-day period produced more malformed embryos and foetuses than that treated with the same dose over a 16-day period. To this end, it is suggested that a subsequent experiment must take into consideration the determination of the concentrations of the drug in the conceptuses and embryos to help explain the findings more clearly. The appropriate biochemical analyses could also be included.

Although the exact mechanisms by which drugs may affect some of the processes of organogenesis, especially neurulation, are not clear, a few suggestions may be made. The poorly developed yolk sac vasculature, sluggish circulation and palor of the red blood cells in the drug-treated groups suggest anaemia as a factor. The anaemia would result in low oxygen levels available to the embryos thereby reducing the oxygen tension in their tissues. Low oxygen tension in the tissues of embryos have been postulated to cause cell degeneration and necrosis leading to

dysmorphogenesis, especially in organs undergoing rapid morphogenesis such as the neural tube (Miki *et al.*, 1988). Determination of the haemoglobin content in future studies could also help substantiate this observation.

Another possibility is that neural tube dysmorphogenesis could be due to the drug's effect on cell-intercellular matrix interactions (Ambroso and Harris, 1993). This is basically because interference with protein synthesis would also affect the production of glycoprotein of the extracellular matrix.

It has been reported that chloroquine crosses the placental barrier in humans (Ullberg *et al.*, 1970) and in rats (Wilson, 1973) and may therefore accumulate in foetal tissues. Although the concentrations of the drug in the embryos and their surrounding tissues were not determined, it is equally possible that chloroquine was able to accumulate in the conceptuses under investigation. According to Padykula (1958), the accumulation of most drugs in embryos could partly, contribute to the formation of abnormalities during the period of organogenesis in the embryos. This he explained could be due to these drugs adversely affecting the balance of isozymes like acid and alkaline phosphatases, lactate and malate dehydrogenases as well as non-specific esterases, which are very critical during this period. Although there was no chloroquine

detected in the blood samples of the pregnant rats after 48 hours of its introduction and also that the concentrations in either the placenta or the embryos were not determined in this study, it was possible the chloroquine accumulated in the tissues, a situation very characteristic of chloroquine. This chemical imbalance caused by chloroquine may also account for the observed dysmorphogenesis in the drug-treated embryos.

Again, direct toxicity caused by possible accumulation of the drug on the embryo could be a debilitating factor. Lindquist and Ullberg (1972) and Dencker *et al.* (1975) had also reported this accumulation. According to Lindquist and Ullberg (1972), this accumulation of chloroquine during the critical morphogenetic and histogenetic period in the embryos probably accounted for dysmorphogenesis in some organ systems such as those of the optic and otic primordia.

With the state of the current findings, some aspects worthy of further investigation are the presence and the concentrations of the drug in the placenta, yolk sac and the embryonic tissues. The form in which the chloroquine was presented whilst in the maternal blood system, as well as after it had crossed the placental barrier to reach the embryos may also be investigated. Histological analyses of the various relevant organ systems are also recommended in future studies. A thorough knowledge of how the drug affects the structural and functional integrity of the visceral

endodermal cells may help explain the process of teratogenic insults in rats in particular, and eutherians in general.

Findings from the recommended investigations will help explain whether the drug actually caused the observed trend and also whether the teratogenic component of the drug was the racemate or its metabolites.

SUMMARY AND CONCLUSION

It can be deduced from the foregoing discussions, that, there exists a correlation between the dosages administered intraperitoneally and the peak concentrations produced in the blood of the non-pregnant female rats. Again, it was shown that the three dosage regimens of the chloroquine diphosphate 7.0 mg/kg body weight, 10.0 5 mg/kg body weight and 12.5 mg/kg body weight administered to 9½-day pregnant Sprague-Dawley rats, even at those relatively low levels, proved to be embryotoxic in terms of growth retardation and dysmorphogenesis.

It was also shown that the levels of growth retardation observed in the present study corresponded generally to the levels of dosages administered and that the degree of growth retardation and dymorphogenesis caused were milder than those observed by Tagoe and Ofori-Adjei (1995).

It may also be said that the drug applied in the present study possibly influenced the embryonic development by altering such fundamental factors as intracellular compartments, surface of the cells, extracellular matrix as well as the entire embryonic environment (Persaud, 1985). These alterations, in effect, possibly resulted in derangements in the form of malformation, disruption, deformation and dysplasia during the intrauterine development of the embryos (Moore, 1992).

Although all the treatment groups produced some abnormalities, the levels of abnormalities were low. The seemingly low rates of growth retardation and dysmorphogenesis produced by the three dosages could be as a result of the ability of the drug to stimulate the parent rats and/or embryos to develop microsomal enzymes in their systems. These enzymes possibly caused the auto-metabolism of the drug thereby avoiding a possible build up to cause any appreciable damages in course of the 24 hr-period.

On the relatively high rate of dysmorphogenesis of about 10% in the controls, however, it is also suggested that the role of the phosphate-buffered saline used as the solvent for the chloroquine must be investigated by the use of a non-injected control alongside the injected ones. This particularly will also help ascertain the roles played by factors like apoptosis and necrosis as well as the attendant individual rates of healing during the period of organogenesis. This will help to determine

the specific role(s) each of these factors played in the observed levels of growth retardation and dysmorphogenesis in all the treatment groups assessed.

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APPENDIX A

WHOLE BLOOD CHLOROQUINE ANALYSIS IN RATS

(CHLOROQUINE ASSAY)

A. STOCK AND WORKING SOLUTIONS

	SOLUTION	CONCENTRATION (µg/ml)
1.	CHLOROQUINE – CQ (STOCK SOLUTION)	50µg/ml
	" " (WORKING SOLUTION)	2.0 µg/ml
	INTERNAL STANDARD (STOCK SOLUTION)	6.0µg/ml
	INTERNAL STANDARD (WORKING SOLUTION)	2.4µg/ml
	DESETHYL CHLOROQUINE (STOCK SOLUTION)	50µg/ml
	DESETHYL CHLOROQUINE (WORKING SOLUTION)	1.0µg/ml

B. REAGENTS AND SOLVENTS FOR EXTRACTION

1. 0.1 Diethylamine
2. 1.0M NaOH
3. Diethylether

C. MOBILE PHASE (COMPOSITION AND FORMULATION)

1. 80% Acetonitrile
2. 19.5% Methanol
3. 0.5% Diethylamine
4. Sonicated (mixture of 1, 2 and 3) for 15-20 minutes

D. SYSTEM SET -UP AND CHROMATOGRAPHIC CONDITIONS

1. Pump: LKB (Broma) 2150 H.P.L.C. Pump
2. Flow rate: 1.0ml/ minute.
3. Injector: Rheodyne
4. Column: Straight Phase
5. Detector: Shimazu RF – 530 Flourescence

   H.P.L.C. Monitor

6. Wavelengths: Emission :- 385nm Extinction :- 335nm
7. Recorder: PM. 8251 Single Pen Recorder
8. Pressure:
9. Sensitivity: High
10. Range: 64/32

PREPARATION OF STANDARD CURVE

RANGE: (100ng 1000ng)

	SOLUTION	VOLUME (MICROLITRES µl)						
1.	CQ - Stock	Blank	100	200	400	600	800	1000
2.	CQ-Working	0	50	100	200	300	400	500
3.	Desethylchloroquine	0	25	50	100	150	200	250
4.	Internal standard (I.S.)	0.075ml / 75ml						
5.	0.1% diethylamine	1.0ml / 1000ml.						
6.	1.0M NaOH	5ml / 500ml.						
7.	Diethylamine	6ml / 6000ml.						

8. Mixed for fifteen (15) minutes.
9. Pipetted off the etheral layer.
10. Evaporated in water bath at temperature between 37°C – 40°C.
11. Reconstituted in 200ml of mobile phase.
12. Injected 20ml of reconstituted solution.

DETERMINATION OF CHLOROQUINE CONTENT IN BLOOD SAMPLES.

1. Proceeded as from steps 4 to 12 above.

TABLE A :VALUES FOR STANDARD CURVE FOR DETERMINATION OF CHLOROQUINE LEVELS IN WHOLE BLOOD OF RATS

Conc(ng/ml)	PEAK HEIGHT(mm)			PEAK HEIGHT RATIO (PHR)	
	CQ	IS	DCQ	CQ	DCQ
0	0	52	0	**0.00**	0.00
100	14	63	20	**0.22**	0.68
200	49	80	43	**0.61**	0.88
400	114	89	70	**1.28**	1.27
600	164	94	113	**1.74**	0.83
800	167	74	129	**2.26**	1.74

CQ : CHLOROQUINE

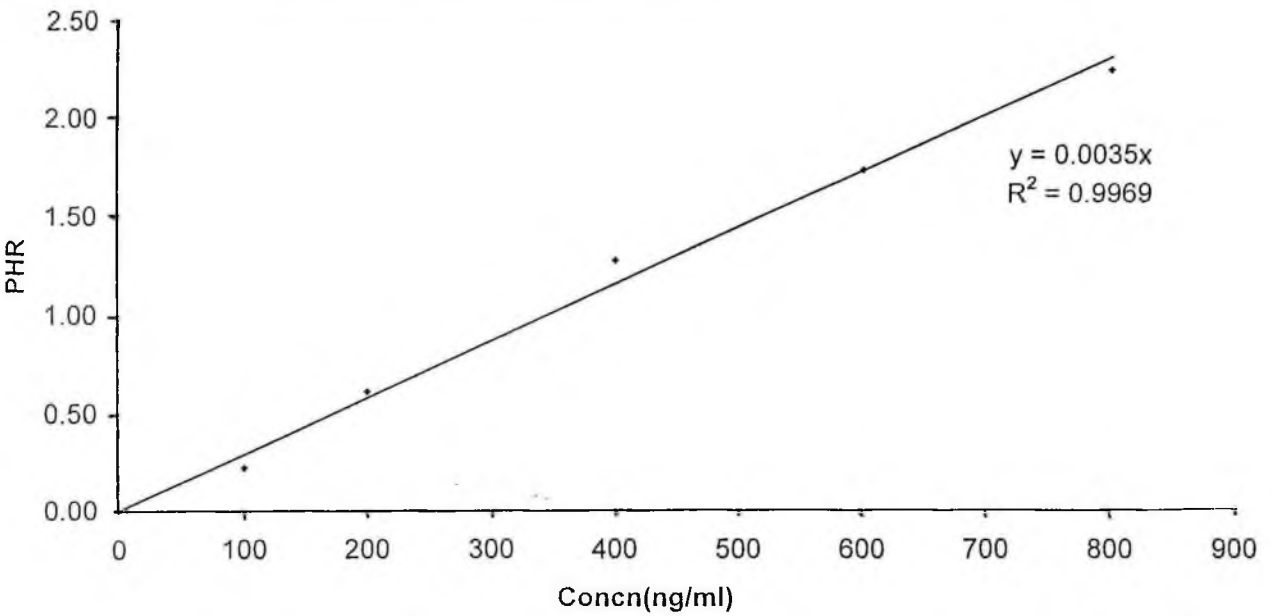
IS : INTERNAL STANDARD

DCQ : DESETHYLCHLOROQUINE

PEAK HEIGHT RATIO OF CQ : RATIO OF CQ TO IS

PEAK HEIGHT RATIO OF CQ : RATIO OF DCQ TO IS

FIGURE : STANDARD CURVE FOR DETERMINATION OF CHLOROQUINE LEVELS IN WHOLE BLOOD OF RATS



APPENDIX B

MODIFIED LOWRY PROTEIN ESTIMATION FOR USE IN DETERMININGEMBRYO PROTEINS IN 1.0M NaOH

Because the various colour development steps are sensitive to pH changes the presence of 1M NaOH in the sample shifts the reaction pH away from its optinum. By neutralising the excess alkali with acid therefore, the buffers in the reagents can control the pH balance effectively.

STOCK SOLUTIONS

A. 1.0M NaOH : 40g/l

B. 3.0M HCl : Concentrated HCl is 11.0M, so 3.67ml of concentrated HClwas made up to 11ml with distill water.

C. Sodium carbonate : 2g anhydrous Na_2CO_3 per 100ml water.

D. Copper sulphate : 1g $CuSO_4$. $5H_20$ per 100ml water

E. Sodium tartrate : 2g $(CH(OH).COONa)_2$. $2H_20$ per 100ml water.

F. Bovine serum albumin (B.S.A.) : 4.58mg BSA/ml dissolved in 1.0M NaOH (stored at -20^oC)

NB. Takes about 1 hour to dissolve at room temperature.

G. Folin - Ciocalteau's reagent : BDH (Stored at 4^oC).

(i) Folin A — Added 1ml of (d) and 1ml of (e) to 100ml of (c). (Mixed and prepared daily.)

(ii) Folin B — Diluted 1: 1 with water.

PREPARATION OF FOLIN B

(FOLIN - CIOCALTEAU REAGENT)

1. 100g sodium tungstate ($Na_2 WO_4, 2H_2O$) and 25g sodium molybdate ($Na_2MoO_4, 2H_2O$) were dissolved in 700ml of water in a 2-litre round-bottomed flask.
2. 50ml of syrupy 85% phosphoric acid (H_3PO_4) and 100ml of concentrated hydrochloric acid (HCl) were added.
3. The product was refluxed for 10 hours using an all-glass apparatus.
4. 150g of lithium sulphate (Li_2SO_4), 50ml of water and a few drops of bromine were added.
5. These were boiled without a condenser for about a quarter of an hour to remove the excess of bromine.
6. It was cooled and made up to one litre with distill water.
7. The solution was then finally filtered ready to be used.
8. (The solution had no greenish tint).

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TOTAL PROTEIN CONTENT DETERMINATION (METHOD)

1. Embryo was placed in 1.0 ml MNa0H in a sealed tube and incubated at 37°C for two (2) hours, whirl -mixing every 10-15 minutes.
2. 0.145ml of 3M HC1 was added and mixed thoroughly.
3. Two (2) aliquots of 0.5ml each of the sample were taken into nine milli-litre (9ml) plastic tubes.

4. 2.5ml Folin A was added to each tube and left at room temperature for (20) inutes.

5. 0.25ml Folin B was then added and then thoroughly **MIXED IMMEDIATELY** after each tube. (This is because seconds delay diminishes the colour reaction).

6. The whole set-up was left at room temperature for forty-five (45) minutes.

7. The absorbance was read at a wavelength of 750nm against a distil water blank.

STANDARD CURVE

0 – 50 ul (in 10 ul aliquots) of BSA stock solution were taken and made up to 1ml with 1M NaOH.

Then proceeded as from stage 2 above.

The resultant amount of standard protein in the 0.5 ml will then range between 0 and 100µg BSA.

A standard curve with a straight line going through the origin was drawn. From the gradient one can calculate from $y = a + bx$, the amount of protein per embryo as follows:

$$\text{Protein content of embryo (ug)} = \frac{c}{\text{gradient}} \times 2.29$$

Where:

c = corrected absorbance (observed - blank)

Blank = 0 µg BSA in standards.

2.29 = 0.5 ml aliquots from 1.145 ml total.

TABLE B: VALUES FOR STANDARD CURVE FOR DETERMIN
TOTAL PROTEIN CONTENTOF EMBRYOS

CONCN	ABS	c ABS
0	0.023	0
10	0.050	0.027
20	0.093	0.074
30	0.129	0.106
40	0.180	0.136
50	0.217	0.169

ABS: ABSORBANCE AT 750 NM

cABS: CORRECTED ABSORBANCE AT 750 NM

APPENDIX C

INJECTION WITH PBS ONLY (CONTROL)

Sample	Abs.	cAbs	TPC(µg)	YSD (mm)	CRL (mm)	HdL (mm)	SOM.	M.Sc.
1	0.560	0.517	348.21	4.3	3.2	2.5	34	23
2	0.612	0.569	383.24	4.5	3.2	2.5	32	25
3	0.673	0.630	424.32	4.1	2.9	2.1	35	25
4	0.606	0.563	379.20	4.0	3.0	2.2	32	27
5	0.640	0.597	402.10	3.9	3.0	2.1	36	28
6	0.519	0.476	320.60	3.8	2.6	1.9	31	27
7	0.507	0.464	312.52	4.1	2.4	1.9	35	28
8	0.650	0.607	408.83	4.2	3.3	2.8	32	27
9	0.748	0.705	474.84	4.3	3.5	2.8	36	26
10	0.631	0.588	396.04	3.6	2.9	2.0	35	30
11	0.604	0.561	377.85	3.7	3.2	2.3	30	26
12	0.665	0.622	418.94	3.9	2.4	1.8	36	27
13	0.718	0.675	454.63	3.7	2.2	1.5	31	25
14	0.655	0.612	412.20	5.3	4.1	2.8	29	33
15	0.605	0.562	378.52	5.2	4.3	2.8	30	34
16	0.504	0.461	310.50	5.0	4.1	2.1	35	33
17	0.448	0.405	272.78	3.2	3.4	2.2	36	31
18	0.364	0.321	216.20	4.6	3.7	1.9	34	35
19	0.516	0.473	318.58	3.6	2.5	1.9	35	35
20	0.408	0.365	245.84	3.2	2.3	1.7	32	32
21	0.386	0.343	231.02	3.2	2.2	1.9	32	28
22	0.520	0.477	321.27	5.0	3.4	2.4	30	28
23	0.480	0.437	294.33	5.3	3.2	2.4	32	25
24	0.638	0.595	400.75	5.0	3.2	2.8	34	30
25	0.468	0.425	286.25	4.8	3.1	2.1	35	26
26	0.409	0.366	246.51	4.7	3.1	2.5	28	22
27	0.511	0.468	315.21	5.2	2.9	2.2	29	21
28	0.494	0.451	303.76	3.6	1.9	1.0	30	24
29	0.493	0.450	303.09	3.5	2.2	1.6	34	32
30	0.633	0.590	397.38	3.9	2.9	2.1	36	31
31	0.486	0.443	298.37	4.0	2.3	1.6	32	29
32	0.490	0.447	301.07	4.2	2.3	1.8	28	30
33	0.863	0.820	552.29	3.9	3.2	2.7	28	31
34	0.596	0.553	372.46	3.7	2.9	2.1	29	34
35	0.535	0.492	331.38	3.6	2.9	2.1	27	33
36	0.586	0.543	365.73	3.8	2.6	1.9	28	29
37	1.270	1.227	826.42	4.2	3.6	2.9	28	34
38	1.390	1.347	907.24	4.6	3.7	3.7	29	35
39	1.280	1.237	833.16	4.6	3.5	2.9	28	33
40	0.448	0.405	272.78	3.2	2.4	1.8	27	34
41	0.687	0.644	433.75	3.9	2.9	2.1	30	29
42	0.792	0.749	504.47	3.6	2.9	2.7	31	28
MEAN			389.40	4.14	2.99	2.22	31.69	29.11
SEM			150.20	0.61	0.56	0.49	2.89	3.79

Number =43.

INJECTION OF CQ AT DOSAGE OF 7.5 MG/KG BODY WEIGHT

Sample	Abs.	cAbs	TPC(µg)	YSD (mm)	CRL (mm)	HdL (mm)	SOM.	M.Sc.
1	0.362	0.342	230.35	5.3	4.3	2.3	34	29
2	0.880	0.837	563.74	5.0	3.9	2.6	34	30
3	0.860	0.817	550.27	5.0	3.5	2.3	32	30
4	0.876	0.833	561.05	5.2	4.5	2.5	35	32
5	0.692	0.649	437.12	5.1	4.2	2.2	38	30
6	0.892	0.849	571.83	6.1	3.3	2.5	35	30
7	0.944	0.901	606.85	4.9	2.9	1.2	34	30
8	0.820	0.777	523.33	3.1	2.5	2.1	35	28
9	0.735	0.692	466.08	4.8	2.6	1.6	35	30
10	0.806	0.763	513.90	4.7	3.9	2.6	31	28
11	0.846	0.803	540.84	5.3	4.5	2.2	35	29
12	0.698	0.655	441.16	5.8	4.8	1.8	39	32
13	0.372	0.329	221.59	4.7	3.6	1.7	34	29
14	0.516	0.473	318.58	3.8	3.3	1.9	32	29
15	0.529	0.486	327.34	3.8	3.2	1.9	32	23
16	0.500	0.457	307.80	3.2	2.8	1	29	20
17	0.521	0.478	321.95	1.8	1.2	1.3	30	17
18	0.432	0.389	262.00	3.5	2.5	1.6	28	22
19	1.225	1.182	796.11	3.8	3.2	1.5	33	21
20	1.250	1.207	812.95	3.6	3.1	1.2	33	21
21	0.425	0.382	257.29	3.2	2.8	1.3	30	24
22	0.525	0.482	324.64	3.4	2.6	1.6	31	26
23	0.536	0.493	332.05	3.7	2.6	1.8	32	24
24	0.504	0.461	310.50	3.3	2.6	2.2	38	24
25	0.428	0.385	259.31	4.6	3.2	1.9	31	32
26	0.362	0.319	214.86	3.5	2.6	1.8	30	33
27	0.902	0.859	578.56	2.5	1.6	1.1	28	28
28	0.532	0.489	329.36	4.3	2.4	1.2	28	31
29	0.632	0.589	396.71	4.2	2.5	1.3	34	32
30	0.274	0.231	155.59	4.0	2.1	1.5	32	32
31	0.286	0.243	163.67	5.0	2.9	1.8	31	34
32	0.316	0.273	183.87	3.9	2.1	1.7	26	30
33	0.286	0.243	163.67	4.2	3.2	1.4	27	35
34	0.312	0.269	181.18	4.3	2.9	1.6	33	31
35	0.423	0.380	255.94	4.6	3.5	2.1	33	34
36	0.633	0.590	397.38	4.8	3.5	2.0	30	33
37	0.324	0.281	189.26	4.7	3.6	1.9	29	33
MEAN			380.23	4.24	3.095	1.789	32.19	28.54
SEM			173.45	0.91	0.80	0.44	3.05	4.44
Number = 39.								

INJECTION OF CQ AT DOSAGE OF 10.0 MG/KG BODY WEIGHT

Sample	Abs.	cAbs	TPC(µg)	YSD (mm)	CRL (mm)	HdL (mm)	SOM.	M.Sc.
1	0.240	0.197	132.7	3.5	2.5	1.3	28.0	29.0
2	0.480	0.437	294.3	3.9	2.3	1.7	28.0	29.0
3	0.590	0.547	368.4	2.8	2.1	1.4	26.0	29.0
4	0.197	0.154	103.7	4.5	3.6	1.3	32.0	31.0
5	0.365	0.322	216.9	3.2	2.6	1.3	30.0	27.0
6	0.556	0.513	345.5	4.9	3.1	1.4	30.0	29.0
7	0.512	0.469	315.9	4.7	3.2	2.1	33.0	30.0
8	0.623	0.580	390.6	4.3	3.1	1.9	29.0	30.0
9	0.297	0.254	171.1	3.9	3.0	1.8	29.0	30.0
10	0.325	0.282	189.9	4.9	3.5	1.7	30.0	28.0
11	0.491	0.448	301.7	3.7	3.1	1.8	35.0	29.0
12	0.336	0.293	197.3	4.2	3.4	2.0	33.0	30.0
13	0.310	0.267	179.8	3.5	2.6	1.6	29.0	31.0
14	0.358	0.315	212.2	3.2	2.5	1.5	29.0	28.0
15	0.216	0.173	116.5	2.8	1.5	1.0	30.0	28.0
16	0.424	0.381	256.6	3.5	2.4	2.1	32.0	29.0
17	0.442	0.399	268.7	4.1	3.4	1.9	34.0	31.0
18	0.263	0.220	148.2	3.4	2.5	1.6	27.0	28.0
19	0.305	0.262	176.5	4.3	3.4	2.7	32.0	29.0
20	0.429	0.386	260.0	3.6	2.1	1.2	31.0	28.0
21	0.270	0.227	152.9	2.8	1.4	1.1	29.0	27.0
22	0.423	0.380	255.9	2.7	1.2	1.1	29.0	27.0
23	0.276	0.233	156.9	2.6	1.5	1.4	31.0	26.0
24	0.344	0.301	202.7	3.1	2.1	1.3	35.0	27.0
25	0.493	0.450	303.1	3.5	2.8	1.8	33.0	28.0
26	0.393	0.350	235.7	2.8	1.9	1.1	30.0	27.0
27	0.190	0.147	99.0	3.1	2.1	1.4	29.0	28.0
28	0.493	0.450	303.1	3.2	2.5	1.9	33.0	27.0
29	0.510	0.467	314.5	3.4	2.6	1.9	32.0	29.0
30	0.262	0.219	147.5	3.3	2.6	1.8	34.0	29.0
31	0.309	0.266	179.2	2.6	1.6	0.9	29.0	29.0
32	0.344	0.301	202.7	3.0	1.4	0.9	33.0	27.0
33	0.540	0.497	334.7	4.8	2.9	1.5	36.0	30.0
34	0.342	0.299	201.4	3.1	2.1	1.3	34.0	26.0
35	0.192	0.149	100.4	2.5	1.6	0.8	31.0	25.0
36	0.233	0.190	128.0	2.4	1.6	0.9	29.0	26.0
37	0.401	0.358	241.1	3.9	2.4	1.6	34.0	27.0
38	0.273	0.230	154.9	3.2	2.6	1.2	33.0	28.0
39	0.31	0.267	179.8	3.1	2.4	1.7	33.0	29.0
40	0.274	0.231	155.6	3.1	2.4	1.5	34.0	27.0
41	0.163	0.120	80.8	2.9	2.1	1.1	32.0	29.0
42	0.168	0.125	84.2	2.9	1.9	1.2	33.0	28.0
43	0.218	0.175	117.9	2.4	1.8	1.0	29.0	27.0
MEAN			208.8	3.4	2.4	1.5	31.2	28.3
			82.0	0.7	0.6	0.4	2.4	1.4

Number = 43

INJECTION OF CQ AT DOSAGE OF 12.5 MG/KG BODY WEIGHT

Sample	Abs.	cAbs	TPC(µg)	YSD (mm)	CRL (mm)	HdL (mm)	SOM.	M.Sc.
2	0.154	0.111	74.76	4.2	3.1	2.4	33	29
3	0.195	0.152	102 38	2.9	2.8	0.9	32	33
4	0.238	0.195	131.34	2.6	1.6	2.1	29	31
5	1.075	1.032	695.08	5.3	4.3	2.0	35	30
6	0.218	0.175	117.87	5.2	4.5	2.2	35	28
7	0.250	0.207	139.42	4.8	3.2	1.6	34	30
8	0.217	0.174	117.19	5.3	3.8	2.3	32	31
9	0.491	0.448	301.74	3.3	2.4	1.3	30	30
10	0.980	0.937	631.10	4.2	3.2	2.1	29	31
11	0.256	0.213	143.46	2.4	1.6	1.0	29	33
12	0.153	0.110	74.09	2.8	2.2	1.3	29	33
13	0.186	0.143	96.31	2.4	1.4	0.9	30	30
14	0.235	0.192	129.32	3.0	1.8	1.2	29	25
15	1.075	1.032	695.08	3.5	2.3	1.2	35	25
16	0.211	0.168	113.15	2.5	1.8	1.1	28	27
17	0.223	0.180	121.24	2.4	1.3	0.8	34	25
18	0.203	0.160	107.76	2.3	1.6	0.9	28	25
19	0.209	0.166	111.81	3.0	1.3	0.9	28	28
20	0.189	0.146	98.34	2.7	1.5	1.0	28	28
21	0.302	0.259	174.44	2.6	1.5	1.1	30	27
22	0.169	0.126	84.86	2.4	1.2	0.8	32	24
23	0.283	0.240	161.65	2.6	1.9	1.1	31	25
24	0.499	0.456	307.13	2 4	1.6	1.0	32	27
25	0.255	0.212	142.79	3.4	2.3	1.3	30	29
26	0.166	0.123	82.84	2.6	1.4	1.0	28	27
27	0.316	0.273	183.87	2.3	1.2	0.9	32	28
28	0.233	0.190	127.97	3.0	1.5	0.8	30	30
29	0.189	0.146	98.34	2.1	1.2	0.9	35	27
30	0.65	0.607	408.83	3.2	2.5	1.8	32	31
31	0.331	0.288	193.98	2.7	2.1	1.2	25	30
32	0.142	0.099	66.68	2.5	1.3	0.8	28	28
33	0.163	0.120	80.82	2.6	1.1	0.9	30	29
34	0.183	0.140	94.29	1.8	1.1	0.7	29	27
35	0.172	0.129	86.89	2.5	1.9	1.1	28	27
36	0.207	0.164	110.46	2.6	1.6	0.8	30	29
37	0.233	0.190	127.97	2.4	1.8	1.0	31	27
38	0.145	0.102	68.70	2.6	1.8	1.1	32	29
39	0.224	0.181	121.91	3.1	2.1	1.6	34	32
40	0.315	0.272	183.20	2.4	1.5	1.0	35	30
41	0.127	0.084	56.58	3.2	2.1	1.4	30	31
MEAN			174.14	3.00	2.01	1.24	30.78	28.65
SEM			160.02	0.88	0.85	0.47	2.52	2.38
Number =41								