Acta Pharmaceutica Sciencia
48: 81-94 (2006)

Chloroquine Accentuates Accelerated Skin Wound Healing and Repairs in Rat

E. Oforah[1*] and S. Asaka,

[1] Department of Clinical Pharmacy and Biopharmacy, Faculty of Pharmacy, University of Uyo, P. M. B. 1017 Uyo, Nigeria.

Abstract

The effect of chloroquine an antimalarial drug on full thickness skin wound healing and repairs was studied in rat. Chloroquine and test drugs were ad ministered for 3 days to rat inflicted with cutaneous skin wound. At Day 5, the indices of healing and repairs:- zinc wound tissue content epithelial healing index, skin tension test and dermal proliferative index were evaluated. Chloroquine increased significantly these indices of wound healing compared with control (normal saline treated) and these effects of chloroquine were quantitatively higher compared with Ascorbic acid and chloroquine + tyrphostin treated wounds. Chloroquine administration accelerated wound healing and repair probably due to its effect in inhibiting lysosomal peptidases which are known to degrade the peptide growth factors (with intrinsic tyrosine kinase activity) and collagens necessary for wound repairs.

Key words: Chloroquine, enhanced skin wound healing and repairs, Rat.

Introduction

We have observed that in some children aged 6-14 yeas old, wounds and bruises healed faster during oral or parental chloroquine prophylactic administration in endemic malaria area compared with children without such chloroquine therapy. Further monitoring of children and young adults showed that wounds healing were enhanced with chloroquine was

* Corresponding author: e-mail: eoforah@yahoo.co.uk

administered oral or parenteral at prophylactic or therapeutic doses (Oforah, unpublished report). Chloroquine is a 4-amino quinoline antimalarial drug that has gained credibility in use despite relentless effort of resistant falciparum malaria (WHO, 1993; WHO; 1991). Various protocols have been devised to sustain the efficacy of the drug during malaria treatment (Bloland et al, 1992; Bloland et al, 1998). Chloroquine has been suggested in other uses such in HIV (Boehart et al 1999; Romanelli et al 2004; Romanelli et al 2006). Chloroquine is a lysosomotropic drug and so inhibits insulin-like growth factors, (Smith et al 1997; Bevan et al 1995; Lappova et al 1996), β-amyloid peptides (Hammand et al, 1997; Mieve et al, 1997) and procollagen Elftheriades et al (1996). Epidermal growth factor (EGF), insulin, insulin-like growth factor (IGF), platelet derived growth factor (PDGF) are growth factors that bind to receptors with integral protein kinase activities. These peptides are targets for lysosomal proteolysis during receptor trafficking, receptor mediated internalization and endocytosis. If these growth factors are protected by chloroquine from lysosomal peptidase mediated degradation then it is reasonable to infer that this may be the mechanism by which chloroquine enhances skin wound healing and repairs. Efficient repairs of cutaneous wound requires a programmed biochemical spatial and temporal series of regulatory events. This complex process s characterized by re-epithelization and restoration of the underlying extra-cellular matrix (ECM) with notable increased synthesis of collagen, proteoglycan and degradative remodeling of the ECM. The migration and proliferation of keratinocytes, endothelial cells, fibroblasts and inflammatory cells to the site of injury are dependent on controlled degradation of the ECM supergene family. EGF is the main growth factor responsible for the acceleration of healing process during epidermal wound repair, (Brown et al 1986, Babul et al, 1996). Insulin promotes the synthesis of proteoglycan, and collagen (Cechowska – Pasko et al, 1996), IGF stimulate the synthesis of collagen by connective tissue cells (Kjellstron et al, 1984). All these situations taken together suggest that an adequate EGF, insulin, IGF, PDGF, and collagen deposition at wound site would be available for wound healing during chloroquine administration. However adequate collagen accumulation depends not only on synthesis stimulated by EGF, insulin, IGF, PDGF, TGF etc. but also on collagen degradation achieved by a family of metalloproteinase which depend on zinc ions for their activity. The aim of this study was to investigate if chloroquine an antimalaria drug will enhance skin wound healing in rat using wound tissue since zinc

content, skin tension test, dermal proliferative index, and epithelia healing index as appropriate parameters reflective of extent of such wound healing and repairs.

Materials and Methods

Chemical, Drugs and Materials

Chloroquine sulphate (Nivaquine, May and Baker, Nigeria) Ascorbic Acid (Sigma USA), Tyrphostin 1478 (Calbiochem, USA), procaine penicillin (Neimeth, Nigeria Ltd.) were all purchased from representative of the companies or the companies themselves. Other chemicals and reagents are analytical grade and sourced commercially. Serohydraulic tension instrument (Instron, USA) was used in tension measurements.

Animals

Pathogen and viral free albino Wistar rats weighing 180-200g were obtained from Vom Veterinary Research centre, Vom, Jos, Nigeria. Animals had free access to (Chow pellets) food and potable water. They were kept single in cages in groups and acclimatized to laboratory environment for 7 days before the experimentations. All procedures were approved by the Animal Care committee of the University of Uyo. We complied and conformed to the guidelines as established in the guide for care and use of laboratory animals in research.

Drug Administration

Chloroquine (10mg kg^{-1} ip); tyrphostin (20μg ml^{-1} ex vivo); ascorbic acid (100mg rat^{-1}, ip) normal saline (100ml kg^{-1} ip), Chloroquine (10mg kg^{-1} ip); + tyrophostin (20ug ml^{-1} ex vivo); were administered differently to rats, inflicted with skin wound, daily for 3 days. There were no mortality at the doses of test drugs employed.

Full thickness skin wound model

The rat dorsal skin was shaved and cleansed with isopropyl alcohol under diethyl ether anesthesia. The skin was linearly incised 2cm long up to the level of subcutaneous adipose tissue on either side of the midline using sterile surgical blade (number 10). The wounds

were closed midway with polygene suture 6/0. A sterile dressing was then placed circumferentially around the truck to protect against infection as a single intraperitoneal dose of procaine penicillin (400 iu/rat) was administered. At the 5th day after the skin wound incision, the animals were prepared for healing assessment.

I. Tissue Zinc Content

Measurement of wound zinc content was performed as previously described Gonul et al, (1998). Briefly, wound tissue were excised and dried overnight at 100°C, weighed and digested in 1.0ml analar grade nitric acid sulphuric acid at ration (3:1) for 2h at 80°C. The samples were diluted 1:10 with deionisied water. Wound zinc content were measured at 123.5mm using Atomic Absorption spectrophotometer (Model Unicam, 919 UK).

II. Epithelial healing index (Percentage of re-epithelization)

Full thickness biopsy was excised across each wound fixed in buffered formalin and embedded in paraffin wax before being cut in thin section 5μm) using microtone (Bright UK 3010). Sections were stained with haemozylin and eosin. The extent of epithelial healing was calculated by measuring the distance between intact epithelia edges divided by the total length of the wound (expressed as percentage of re-epithlization).

III. Skin Tension test

The method of Chakravaiti et al (1998) was adopted. Briefly, tensile samples were prepared from the dorsal skin of both normal and wound inflicted rats. The skin was harvested and tested within 1h of sacrifice. Thickness measurements were taken on a small shaved area of the skin using a micrometer. Test samples were produced using a template with gauge region of 100mm wide and 20mm long. The axes of the samples were in line with the superior – inferior direction of the rat. The samples were placed in an alignment jig, the ends being damped. The instrument was loaded to failure in tension at a constant rate of stress. Tensile strength was determined by normalizing the change in actuator displacement for initial sample length. The variations using the instrument was less than 1% for 5 measurements.

IV. Dermal proliferative index

Microtone cut thin slices (5μm) of wound treated sections were stained using mouse monoclonal 1g 12a antibody (Zymed lab, USA). The slices were counter stained with haemoxylin and eosin. The dermal proliferative index was determined by counting the number of proliferative cells nuclear antigen (PCNA) divided by the total number of fibroblasts. The counting was determined by random evaluation of three different (40x) field views of dermis of each sample. The proliferative index was calculated as

$$\frac{\text{PCNA positive nuclei}}{\text{Total fibroblast number}}$$

At least 300 cells around the entire circumference of the dermis were thus evaluated.

V. Data Analysis

All Statistical evaluation of the difference between results from chloroquine, ascorbic acid, tyrphostin, tyrphostin and chloroquine, normal saline were assessed using analysis of variance. Differences between results were analyzed in Tukey HSD post-test or Mann Whitney U test as appropriate.

Result and Discussion

The results of the effect of chloroquine administered intraperitonealy to rats inflicted with cutaneous incision wound are as depicted in Table 1. Chloroquine administration elicited profound healing and repairs based on the parameters used to assess incision wound healing and repairs: - skin zinc content, skin tensile strength, epithelial healing index (percentage of reepithelization) and dermal proliferative index.

Table 1: The Effect of Chloroquine on Wound Healing and Repairs in Rat

	PARAMETERS OF HEALING			
Treatment	Wound Zinc Content (μg^{-1} dry weight)	Tensile Strength (Ncm-1)	Extent of Healing (% of Re-epithelization	Dermal Proliferative Index
(a) Normal rat skin (control)	160 ± 28	3.80	100	100
(b) Skin wounds				
(i) Normal Saline (10ml kg-1) control II	118, ± 28*	0.9 ± 28*	118 ± 0.3*	20 ± 6%*
(ii) Chloroquine (10mg kg^{-1} ip) + Tyrophostin (20µ ml-1 ex vivo	147 ± 28*	1.2 ± 0.4 ††	45 ± 8%†*	35 ± 9%*
(iii) Chloroquine (10mg kg^{-1} ip)	244 ± 41†*	2.15 ± 0.35†	90 ± 10% ††	50 ± 12%††
(iv) Tyrophostin (20µ ml^{-1} ex vivo	120 ± 15*	1.0 ± 0.2*	20 ± 9%	16 ± 8%
(v) Ascorbic acid (100mg/rat ip)	189 ± 10 †*	1.80 ± 0.15*	47 ±120*	38 ± 7% †

Values are mean ± Standard error (N = 6) in triplicates of assays.

*$p<0.05$, significantly different from Control 1 (Normal Skin)

†$p<0.05$, significantly different from Control II (Normal Skin)

††$p<0.01$, significantly different from Control I (Wound Skin)

The effect of chloroquine on skin wound healing and repairs was efficacious and superior to ascorbic acid (a) drug often adjudged to aid skin wound healing in all facets of healing parameters and values were statistically significant. Tyrphostin, an inhibitor of epidermal growth factor receptor tyrosine kinase (Yaish et al, 1988) has low values of the parameter healing indices demonstrating very poor healing and repairs as the control II (normal saline treatment). However co-administration of chloroquine and tyrphostin, improved healing assessment, when compared within. The differences between values of chloroquine + tyrphostin and values obtained from tyrphostin treated wounds were statistically significant. Ascorbic acid treated wounds showed significant healing and repair parameters assessment compared with the normal rat skin. However, the dermal proliferative index from ascorbic acid treatment though 30 $\pm$ 8%, yet statistically significant when compared with control II. ($P<0.05$).

Wound Skin Zinc Content

Values of wound zinc content at Day 5 from chloroquine treated wounds (244 $\pm$ 5 μg dry weight), and ascorbic acid (189 + 10ug dry weight) were statistically significant compared to control. Tyrphostin treatment had zinc value 120$\pm$ 15ug dry weight which was not different from control II. The zinc skin content values obtained from chloroquine and ascorbic acid were statistically significant compared with control I, and control II. There qualitative different zinc values are indication that these drugs have marked effects on biochemical processes that were involved in skin wound healing and repairs. Zinc is a cofactor for metalloproteinase activity in the degradation of the extra-cellular matrix components (Sudbeck et al 1994). Tissue wound zinc is an index of metalloproteinase activity. Some authors have reported of maximal metalloproteinase activity in RAT on Day 5 after wound incision (Gonul et al 1998), so the zinc values which we evaluated on Day 5 of the study will also reflect the maximal metalloproteinase activity. Collagenase subfamily of metalloproteinase requires zinc in its degradation of collagen. As chloroquine treatment resulted in high skin wound zinc content compared with other treatments. These differences of skin zinc wound values may indirectly be from the growth factors – epidermal growth factors, insulin, insulin-like growth factor, platetet derived growth factor, etc and collagen. The low wound zinc value of the tyrphostin, a potent specific epidermal growth factor

receptor tyrosine kinase inhibitor and an improved moderate wound zinc value when co-administered with chloroquine, reinforces chloroquine implication in wound healing. If chloroquine, inhibited intracellular Iysosomal degradation of these growth factors and collagen, then the activities of these growth factor and collagen during wound healing and repairs wound be upgraded. In essence, adequate availability of these growth factors and collagen will stimulate series of biochemical evens including pro-collagenase synthesis. Collagen and soluble factors induce collagenase expression keratinocytes via the epidermal growth receptor (Picher et al, 1999, Sudbeck et al, 1994) Epidermal growth factor family upgrades matrix metalloproteinase (collagenase) synthesis, tyrphostin will depress this vital synthesis. Thus the up-regulation of the growth factors and collagen could be linked to the increase tissue wound zinc content collagens. This process ensures adequate supply of collagen at the wound site.

The migration of cells re-modeling of tissue during wound healing and repairs require the controlled degradation by collagenase of collagen of the extra-cellular matrix and the activation or release of growth factors. (Pessa et al, 1987).

Dermal Proliferative Index

Values obtaining from chloroquine treated wound 50 $\pm$ 8% is statistically significant compared with control II and more than twice the values from other drugs. The significantly higher value from chloroquine treated rat may be connected with increased deposition at wound site for the upgraded activities of the growth factors and collagen because chloroquine upgrades insulin degradation.

Tyrphostin treated rats showed low dermal proliferative index (16 $\pm$ 8%). The tyrphostin effect could have been due to inhibition of the EGFR mediated signal transduction thereby limiting metalloproteinase synthesis and activity, cell migration and collagen degradation. Chloroquine + tyrphostin co-treated wounds have an average dermal proliferative index value 35 $\pm$ 2%. This effect of chloroquine may either be that it blocked the activity of tyrophostin directly or inhibited the lysosomal enzymes activity or has upgraded other growth factors as compensatory to the tyrphostin inhibiting activity of the EGFR signal transduction.

Skin Tensile Strength

Measurement of skin tensile strength of skin wounds reflects the extent of healing and repairs (Killic et al, 1994; Kahlsson et al, 1960). Therefore, the wound skin tensile strength will be higher where the healing process showed excellent healing and repair. Chloroquine treatment showed a significant higher skin tensile strength compared with control II and there comparable with wound treated with normal saline control II. Though the healing effect is superior to control II, its effect is not comparable to control I.

Fibrillar collagens constitute the bulk of connective tissues and provide the biomechanical support for optimal functioning of tissues. Collagen matrix strength is biomechanical support for optimal functioning of tissues. Collagen matrix strength is dependent on it's association with glycoproteins (such as fibronectin, laminin) and proteoglycan. Insulin and insulin-like factors stimulate both synthesis of collagen and proteoglycan. EGF ex vivo enhances epithelization and accumulation of granulation tissue cells, collagen and proteogycan in wound models (Gonul et al, 1998). EGF and Zinc have also be reported to increase the re-epithelization and skin tensile strength of healing wounds by an enhanced deposition of collagen at site of the wound (Karcioglu and Sarper, 1996).

Extent of Healing (% of re-epithelization)

Chloroquine treatment showed 90 $\pm$ 10% extent of healing, ascorbic acid at 72 $\pm$ 12% and chloroquine + tyrphostin at 45 $\pm$ 6%. However wounds treated with normal saline and tyrphostin closed at an average of 24 $\pm$ 10% and 20 $\pm$10% and 20 $\pm$ 10% respectively showing the poor healing effect of normal saline and tyrphostin. In situ hybridizaion showed that collagenase – 1- MRNA was expressed by migrating keratinocytes during re-epithelization and that extracellular matrix and soluble factors induce collagenase – 1 – MRNA in keratinocytes via epidermal grow factors (Pilcher et al, 1999). The closure of cutaneous wounds involved re-epithelization, connective tissues 9collagen) deposition and contraction. Wound contraction brings the margins of open wounds together (Clark 1996), and is mediated largely by platelets. The Epidermal growth factors receptor activation initiates a series of processes including activation of intrinsic EGFR tyrosine kinase a utophosphorylation and the assembly of active signaling complexes at the plasma membrane. Concomitantly receptor trafficking is initiated and the receptor is ultimately delivered to the

lysosome where it is degraded. It could be that 90% healing effect observed with chloroquine treatment could have been due to inhibitory effect of chloroquine on lysosomal mediated degradation of growth factors as they operate similar receptor trafficking. Thus, there would be accentuation of the signal transduction of the critical growth factors and at the same time prevents intracellular procollagen being synthesized from the lysosomal degradation. Ascorbic acid ensures the availability of stabilized synthesis of collagen at the wound site which would be required for re-epithelization Poor healing effect as measured by low percentage of re-epithelization on wound treated ex vivo with tyrophostin could be explained by the inhibition of EGFR signal transduction, as EGF receptor is a downstream intergrin activation in the signal transduction pathway leading to fibroblast migration (Li et al 1999). However, the combination of chloroquine and tyrphostin treatment improved healing probably due to chloroquine effect either blocking the inhibition of tyrphostin or on the lysosomal enzymes or by both mechanisms.

In conclusion, taking these results together demonstrate that skin wound healing and repairs effects of chloroquin may be adduced or probably related to its inhibition of lysosomal enzymes which would have degraded the critical growth factors and collagens. These actions of chloroquine on skin wound healing and repairs parameter is novel and may provide new therapeutic roles apart from its other indications, malaria, lumpus erythematosus, anti-rheumatism and hepatic amoebiasis and currently being assessed in HIV therapy.

References

Babul A ,Gonul B, Ozogal C, Dincer E, Endogen D, Pinar L,N (1996) Epidermis Growth Factor Accelerates Mice Wound Healing. Prof. 8th International Pharm Technol Symposium (IPTS-96) SEPT 9-10, Ankara Turkey.145-146.

Bloland P B, Kazemba PN, Oleo AL, Himanaga B, Barat LM, Rue Bush TK (1998) Chloroquine in Africa, Critical Assessment and Recommendations for Monitoring and Evaluating Chloroquine Therapy Efficacy in Sub- Sahara Africa *Trop Medicine and Int. Health* 3, 543-545.

Bloland PB, Lacking EM, Kazemba PN, Were I Stekette PW, Campbell C (1992) Beyond Chnloroquine Implication of Drug Resistance for Evaluating Malaria Therapy Efficacy and Treatment Policy in Africa *J Infection Diseases* 167,204 -213.

Bevan AP, Christen JR, Tilcerpae J, Smith GD (1995) Chloroquine Augment the Binding of Insulin to its Receptors *Biochemical* J 311,789-795.

Boelart JR, Sperber K, Piette J (1999) Chloroquine Exerts an Additive in Vitro Anti-HIV Type1-effat when Associated with Didanosine and Hydroxyurea *AIDS RRS Human Retrov* 15,1241-1247.

Brown GL Cwitsinger L, Brightwell JR, Ackermen DM Tobin GL, Poll HE, Nascimento CG, Vanlenuella P, Schuty GS (1986) Enhancement of Epidermal Regeneration by Biosynthetic Epidermal Growth Factor, *J Exp. Med.* 163, 1319-1320

Cechowska-Pasko M, Palka J, Bankowski E (1996) Decrease in the Glycosaminoglycan Content in the Skin of Diabetic Rat. The Role of 1GF-1, 1GF Binding Proteins and Proteolytic Activity *Molecular Cell Biochemistry* 154, 1-8.

Clark PAF (1996) in the Molecular and Cellular Biology of Wound Repairs (Clark PAF ed) 2nd Edition 3-50, Plenum Press, NY USA.

Eiftheriades EG, Ferguson A, Sprdages MC, Sarnarel AM (1996) Polyhydroxyleton Regulates Intracellular Procollagen Degradation in Cultured Rat Cardiac Fibroblast. *J Mol Cell Cardiology* 27 (i)1459-1473

Gonii B, Soylemezogy A, Babal A, Celebi N (1998) Effect of Epidermal Growth Factor Dosage Forms on Micefull Thickness Skin Wound Zinc Levels and Relation to Wound Strength *J Pharm Pharmacology* 50.641-644.

Kjellstrom S, Malmquist J (1984) Insulin Effects on Collagen and Proteins Production in Cultured Human Skin Fibroblasts from Diabetic and Non –Diabetic Subjects *Hormone Metabolic Research* 16,168-71.

Kahlsson G, Nilsson K, Rosengran E (1960) Wound Healing as Dependent on Rate of Histamine Formation *Lancet* 30,230-234.

Killc N, Gonul B, Aidemir H, koz M (1994) Increase in Acid Proteins and Breaking Strength During Wound Healing *J Fac Phar Gazi, Gueda* 11,91-92.

Hammad SM, Ranganathans, Loukinova E, Twal WO, Argraves SW (1997) Interaction of Apolipoprotein J –Amyloid Compelx with Low Density Lipoprotein Receptor Related Protein-2/megalen A Mechanism to Prevent Pathological Accumulation of amyloid β Peptide, *J Biol Chem* 272 (30) 18644-49.

Laato M, Niinikoshi J ,Gerdin B, Lebel L (1980) Stimulation of Wound Healing by Epidermal Growth Factor *Annals Surgery* 203.329-381.

Lappova YL, Leibush BIV, (1998) Receptor Mediated Endocytosis of Insulin in Lower Vertebrates Internalization and Intercellular Preening of 1281 in Isolated Hepotocytes of Lamprey and Frog. *Gen Comp. Endocrinology* 100 (i)-9.

Li J, Lin M-L, Wiepz GT, Guad Arrana AG, Bertics PJ (1999) Intergrin Mediated. Migration of Murine 882 Fibroblast is Dependent on the Expression of an Instant Epidermal Growth Factor *J Biol Chem* 274, 11209 -11219.

Mieve JG, Murphy MP, Marital, Bengualaid, KM, Ivygo (1997) Chloroquine Administration in Mice Increase β Amalyliod Immuno Reactivity and Attenuates Kainate Induced Blood Brain Dysfunction *Neuro Sci Lett* 227,169-172.

Pilcher BK, Dumis J, Schwarty W, Mast, BA, Schultz GS Parks WC, Welgus HE (1999) Keratinocyte Collagenase Expression Requires an Epidermal Growth Factor Receptor Autocrime Mechanism *J Biol Chem*. 274, 10372-10381.

Pessa MC, Blaud KI, Copeland EM (1987) Growth Factor and Determinants of Wound Repair *J Surg Res*. 42, 207-217.

Romanelli F, Hoven AD (2006) Chloroquine and Hydroxychloroquine as Inhibitors of Human Immuno Deficiency (HIV – 1) Activity *Current Pharm. Design* 12, 1121-1127.

Romanelli F, Smith KM, Hoven AD (2004) Chloroquine and Hydoxy Chloroquine as Inhibitors of Human Immuno Deficiency Virus (HIV-1) Activity *Current Pharm Design* 10, 2643-2648.

Sudbeck BP, Pak WC, Welgug HG, Pentland AP (1994) Collagen Mediated Induction of Keratinocyte. Collagenase is Mediated by Tyrosine Kinase and Protein Kinase C. Activities *J. Bul Chery* 269, 30022-20029.

Schultz GS, White M, Mitchell R, Brown G. Lynch J, Twardack DR, Todaro G1 (1987). Epidermal Wound Healing Enhanced by Transforming Growth Factor and Vaccinia Growth Factor *Sciencia* 16, 300.

Smith EP, LU-L, Chernausek SD, Klein DJ (1994) Insulin-like Growth Factor Binding Protein (IGFBP-3) Concentration in Rat Sertoli Cell Condition Medium is Regulated by a Pathway Involving Association of IGFBP-3 with Cell Surface Proteoglycan. *Endocrin* 135, 359-364.

Taish P, Gasit A, Gilon C, Leviski A (1988) Blocking of EGF Dependent Cell Proliferation by EGF Receptor Kinase Inhibitors *Science (Washington, DC)* 242, 933-935.

WHO (1993) Chemotherapy of Malaria and Resistance to Anti-Malaria. Geneva *WHO Technical Report Services* No 529.

WHO World Malaria Situation in 1991 *Part I Weekly Epidemiological Round* 1993, 71, 17-22.

Received:14.10.2004
Accepted:15.03.2005