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Influence of Chloramphenicol and Amoxicillin on Rat Liver Microsomal Enzymes and Lipid Peroxidation

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ABSTRACT

Generation of reactive oxygen species beyond the antioxidant capacity of biological system has been reported to give rise to oxidative stress which through a series of events deregulates cellular functions, leading to oxidative damage and various pathological conditions. This study examined the effect of chloramphenicol and amoxicillin on liver microsomal enzymes Ca^{2+}-ATPase and Glucose-6-Phosphatase (G-6-P) and lipid peroxidation in rats. Male Wistar strain rats weighing 120 – 195 g were divided into four groups. Group one, the control group, received physiological saline, group two received Amoxicillin at 10.71 mg/kg, group three received Chloramphenicol at 28.57 mg/kg, while group four was administered combination of chloramphenicol and amoxicillin. Drugs were administered for ten days and the animals sacrificed on the eleventh day. Detection of oxidation in liver microsomal fraction was carried out by assessment of lipid peroxidation and conjugated diene. Ca^{2+}-ATPase and G-6-P activities and total protein content were also measured. Data were analysed by ANOVA and Student's T-Test. Significant ($p<0.05$) decreases in G-6-P activity by 55.30%, 38.37%, 55.30% and Ca^{2+}-ATPase activity by 38.99%, 30.16%, 26.88% were recorded with chloramphenicol, amoxicillin and chloramphenicol/amoxicillin treatments respectively when compared with the control group while total microsomal protein content was depleted by 70.50% 79.27%, 75.87% respectively. TBARS and Diene Conjugation were significantly ($p<0.05$) elevated in the treated groups. Findings from this study suggest that Chloramphenicol and Amoxicillin induced oxidative stress in rats and perturbed Ca^{2+} homeostasis presumably due to generation of free radicals.

Key words: Chloramphenicol, Amoxicillin, Lipid peroxidation, Ca^{2+}-ATPase, Glucose -6-phosphatase.

INTRODUCTION

The protective roles and curative properties of drugs in man and animals cannot be under-estimated but at the same time, several drugs used in the treatment of human diseases exert varied degrees of toxicities or side effects by precipitating to other pathologies, damaging vital organs and tissues (Aruoma *et al*., 1989; Evans *et al*., 1994; Chang and Schiano, 2007; Andrade and Tulkens, 2011). Metabolism of xenobiotics, drugs inclusive, could lead to their bio-transformation to more reactive substances which are injurious to cells and cellular contents. Also, they could give rise to generation of free radicals and reactive species which have been implicated in many pathological conditions and diseases (Chirino

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and Pedraza-Chaverri, 2009; Tarantino, 2009; Sawicka *et al*., 2013).

Many toxic drugs exerts their toxicity by forming reactive electrophilic intermediates and free radicals which covalently binds to or reacts with nucleophilic sites or molecules in the cell, including proteins, thiols, glutathione (reduced) and nucleic acid giving rise to cellular dysfunction, oxidative stress to the cell and ultimately cell death, either necrotic or apoptotic (Orrenius *et al*., 2011; Wang, 2014). Binding of these substances to thiol-containing enzymes, for example, Ca^{2+}-ATPase could also lead to loss of enzyme activity (Hu *et al*., 2010).

Chloramphenicol, derived from the bacterium *Streptomyces venezuelae* is a broad spectrum antibiotic that was first synthesized on a large scale. It is a bacteriostatic drug that hinders bacterial growth by binding to the 50S subunit of the 70S ribosome of the bacterial and preventing peptide bond formation by inhibiting the peptidyl transferase activity of the bacteria ribosome, thus inhibiting protein synthesis (Balbi, 2004; Barnhill *et al*., 2012). Due to the lipid solubility of Chloramphenicol, it readily traverses the plasma membrane to enter sensitive cells by active transport. It is well distributed throughout the body with about 60% bound to plasma proteins in adults (Laferriere and Marks, 1982).

Chloramphenicol is effective against a wide variety of microorganisms. It is widely used in many parts of the world, especially in the developing countries for the treatment of life-threatening infections such as typhoid fever and meningitis (Turton *et al*., 1999). It exerts mainly a bacteriostatic effect on a wide range of gram-positive and gram-negative organisms and is active against Rickettsia, Chlamydia (psittacosis-lymphogranuloma organisms), and Mycoplasma. It is also indicated in severe salmonella infections and is regarded as an alternative agent for pneumococcal, meningococcal and the ampicillin-resistant *Haemophilius influenza*e infection (Holt *et al*., 1993).

Amoxicillin is a β-lactam moderate spectrum bactericidal antibiotic used in treating various bacterial infections. It is a p-hydroxy derivative of ampicillin effective against certain gram-positive and negative organisms. Amoxicillin which is better absorbed than other β-lactam antibiotics is one of the most common antibiotics prescribed for children in the treatment of a number of infections including pneumonia, skin infections, urinary tract infections, streptococcal pharyngitis and lyme disease among many others. Amoxicillin is often combined with clavulinic acid, a β-lactamase inhibitor to increase its effectiveness due to the susceptibility of amoxicillin to degradation by β-lactamase producing bacteria which are resistant to β-lactam antibiotics. Amoxicillin is widely distributed at varying concentrations in body tissues and fluids. About 20% is bound to plasma proteins with half live of 1 to 1.5 hours (Adam *et al*., 1982). Amoxicillin readily crosses the placenta, while little amount are excreted in breast milk and little amount passing into the CSF.

Various side effects and diseases have been encountered and reported with the use and administration of various antibiotics. Chloramphenicol administration is associated with quite a number of adverse effects. The most serious adverse effect is bone marrow toxicity which manifests in two forms; bone marrow suppression, a usually reversible direct toxic effect and aplastic anaemia, a rare but generally fatal idiosyncratic effect (Cruchaud *et al*., 1963; Krakoff *et al*., 1955; WHO, 1988) which informed mainly the ban of the drug in many countries (Wurtz, 1986). Other adverse effects include: leukemia, Gray baby syndrome, leucopenia, thrombocytopenia, optic neuritis and anorexia (Holt *et al*., 1993; Robin *et al*., 1981; Yunis, 1989; Turton *et al*., 2006). Although the use of chloramphenicol has reduced in many developed countries, it is still very much in use in many developing countries even in spite of its known adverse effects due to its effectiveness, availability and low cost (Farombi *et al*., 2002).

Also, amoxicillin has been shown to exhibit side effects as hypersensitivity reactions such as uticaria, fever joint pains, diarrhoea, anaphylactic shock, erythemateous rashes, chronic lymphatic leukaemia and gastrointestinal irritation. Several workers have associated the hepatotoxicity of amoxicillin-clavulanic acid to the clavulanic acid, a beta lactamase inhibitor and not amoxicillin (Herrero-Herrero and García-Aparicio, 2010; Studniarz *et al*., 2012). Chloramphenicol, a bacteriostatic antibiotic and amoxicillin, a bactericidal antibiotic are occasionally combined to treat severe cases of infection, for example in the treatment of ampicillin resistant Haemophilus influenza. The influence of these drugs on lipid peroxidation, at microsomal level and their effects on membrane bound Ca^{2+}-ATPase and glucose-6-phosphatase as indices of oxidation are the objectives of this study.

MATERIALS AND METHODS

Chemicals: Chloramphenicol and Amoxicillin were obtained from Smithkline Beecham, sucrose, sodium azide (NaN_3) from Fisher Biotech, USA, tris base, potassium chloride (KCl), glucose 6-phosphate, ATP, EDTA, maleic acid, trichloroacetic acid (TCA), thiobarbituric acid (TBA), ascorbic acid, disodium

hydrogen phosphate (Na_2HPO_4), ethyleneglycol-bis-2-aminotetra acetic acid (EGTA) were all purchased from Sigma Chemical, London, while ammonium molybdate, ferrous sulphate ($FeSO_4.7H_2O$) and hydrogen peroxide (H_2O_2) were purchased from BDH, Poole, England. All other reagents were of analytical grade.

Animals: Male and female Wistar strain rats weighing between 120 and 195 g were purchased from the Central animal House of the Faculty of Basic Medical Sciences. Animals were kept in well ventilated cages at temperature between 28-30 ^{0}C and were maintained on normal laboratory chow (Ladokun Feeds, Ibadan, Nigeria) and water *ad libitum*. The rats were handled and treated in conformation to the guidelines of the National Institute of Health (NIH publication 85-23, 1985) for laboratory animal care and use. Animals were allowed to acclimatize for two weeks before the commencement of drug administration.

Experimental design: Rats were randomly divided into four groups. The first group served as the control and received physiological saline. Second group received Amoxicillin at a dose of 10.71 mg/kg body weight/day. The third group received Chloramphenicol at a dose of 28.75 mg/kg body weight/day while Group 4 received both Chloramphenicol and Amoxicillin. Drugs were administered to the animals by oral intubation for 10 days and were sacrificed on the 11th day after an overnight fast.

Preparation of tissue: Experimental animals were sacrificed by cervical dislocation. The livers were quickly removed, rinsed in ice cold 1.15% KCl solution and weighed. The liver samples were chopped and homogenized in 4× liver weight volume of homogenizing buffer using Potter-Elvehjem homogenizer with a loose fitting pestle. The homogenates were centrifuged at 10,000 g, 4 ^{0}C for 15 mins in a Sorvall RC-5B centrifuge. The post nuclear supernatant was further centrifuged at 10,500 g, 4 ^{0}C for 55 mins in a Beckman L-8-80M ultracentrifuge and the resultant pellet was weighed and resuspended in the incubation buffer – 130 mM KCl/ 20 mM Tris, pH 7.2 to a volume of 1 ml/g weight. The preparation was stored at -80 ^{0}C until required.

Biochemical assays: Protein content of the microsomal fractions of the livers was determined by the Biuret method (Gornall *et al*., 1949) using bovine albumin as standard. Glucose 6-phosphatase activity was measured according to the method of Agerbo *et al*., (1992) based on the release of inorganic phosphate. The amount of phosphate released was determined as described by Fiske and Subbarow (1925). Ca^{2+} ATPase activity was assayed by measuring the rate of release of inorganic phosphate from the γ position of ATP spectrophotometrically using the method of Raess and Vincenzi (1980) based on the procedure of Fiske and Subbarow (1925). Membrane Lipid peroxidation was assessed as an index of oxidative stress by measuring the formation of Thiobarbituric acid reactive substances (TBARS) according to the method of Rice Evans *et al.*, (1986). Assessment of conjugated dienes was carried out according to the method of Klein, (1970).

Statistical analysis

Results were expressed as means ± S.D. Data were analyzed by one-way ANOVA and Student's T test. Values were considered statistical significant when $p< 0.05$

RESULTS

Results from this experiment revealed the toxicological effects of both single and co-administration of chloramphenicol and amoxicillin to rats for a period of 10 days at therapeutic doses of 28.75 mg/kg body weight/day and 10.71 mg/kg body weight/day respectively. Table 1 projects the changes in the body weights of the animals treated with both drugs when compared with the control animals. Highest % weight loss was observed in rats co-administered with both chloramphenicol and amoxicillin (12.50±0.87%) followed by the group administered CAP (5.46±2.38%) and then amoxillin treated group (4.62±3.02%).

Table 1
Effect of Chloramphenicol and Amoxicillin treatments on body weight of rats

Treatment	Average Loss in Body weight (g)	% Weight Loss
Control	**3.67 ± 1.24**	**2.71 ± 1.24**
Amox	**8.33 ± 5.43***	**4.62 ± 3.02***
CAP	**10.66 ± 4.64***	**5.46 ± 2.38***
CAP/Amox	**16.67 ± 1.69***	**12.50 ± 0.87***

CAP = Chloramphenicol, Amox = Amoxicillin
**p<0.05 when compared with control.*

Table 2 shows the decreases in microsomal protein content in the drug-treated rats when compared with the control rats. Administration of Amox gave rise to a decrease of 7.00±0.57; CAP gave rise to 6.23±0.77 and CAP/Amox, a decrease of 6.7±1.08 compared to

8.83±4.37 mg protein/ml concentration in the control group. Effect of Chloramphenicol and Amoxicillin treatments on microsomal Glucose-6-phosphatase in rats is presented in Table 3 while Table 4 shows the effect of the drugs on Ca^{2+}-ATPase in rats. The activities of these enzymes were significantly ($p<0.05$) depleted in the drug-treated groups when compared with the control group. The effect on Glucose -6-phosphatase was more pronounced in the Amox-treated group while depletion of Ca^{2+}-ATPase was found highest Chloramphenicol/Amoxicillin -treated group.

Table 2
Effect of Chloramphenicol and Amoxicillin treatments on liver protein content in rats

Treatment	Protein Content (Mg Protein/ml)
Control	**8.83 ± 4.37**
Amox	**7.00 ± 0.57**
CAP	**6.23 ± 0.77***
CAP/Amox	**6.70 ± 1.08***

CAP = Chloramphenicol, Amox = Amoxicillin
**p<0.05 when compared with control*

Table 3
Effect of Chloramphenicol and Amoxicillin treatments on microsomal Glucose-6-Phosphatase in rats

Treatment	Pi (µmol/mg Protein/min)
Control	**0.09 ± 0.001**
Amox	**0.04 ± 0.02***
CAP	**0.05 ± 0.01***
CAP/Amox	**0.05 ± 0.01***

CAP = Chloramphenicol, Amox = Amoxicillin
**p<0.05 when compared with control.*

Table 5 reports the significant ($p<0.05$) induction of lipid peroxidation measured as Thiobarbituric acid reactive substances (TBARS) in Chloramphenicol, Amoxicillin and Chloramphenicol/Amoxicillin treated groups with Chloramphenicol/Amoxicillin group showing highest level of peroxidation when compared with the control group. Similar pattern of lipid peroxidation induction were observed with and without addition of oxidants. Table 6 expresses the effect of Chloramphenicol and Amoxicillin treatments on the formation of conjugated dienes in rat's microsomes. Significant increase ($p<0.05$) in diene conjugation was observed in the drug-treated groups when compared with the control groups with the highest value observed in the chloramphenicol treated group

Table 4:
Effect of Chloramphenicol and Amoxicillin treatments on microsomal Ca^{2+}-ATPase in rats

Treatment	Pi (µmol/mg Protein/min)	Ca^{2+}-ATPase % control
Control	**0.09 ± 0.02**	**100.00**
Amox	**0.03 ± 0.02***	**30.16***
CAP	**0.04 ± 0.02***	**38.99***
CAP/Amox	**0.02 ± 0.02***	**26.88***

CAP = Chloramphenicol, Amox = Amoxicillin
**p<0.05 when compared with control.*

Table 5
Effect of Chloramphenicol and Amoxicillin treatments on Lipid peroxidation

Treatment	With Oxidant (µmol MDA/mg protein)	Without oxidant (µmol MDA/mg Protein)
Control	**0.31 ± 0.01**	**0.18 ± 0.05**
Amox	**0.52 ± 0.07***	**0.38 ± 0.02***
CAP	**0.59 ± 0.01***	**0.37 ± 0.08***
CAP/Amox	**0.60 ± 0.14***	**0.38 ± 0.01***

CAP = Chloramphenicol, Amox = Amoxicillin
**p<0.05 when compared with control.*

Table 6
Effect of Chloramphenicol and Amoxicillin treatments on microsomal Conjugated diene in rats

Treatment	Mean Absorbance at 233 nm
Control	**0.01 ± 0.01**
Amox	**0.24 ± 0.03***
CAP	**0.52 ± 0.35***
CAP/Amox	**0.25 ± 0.03***

CAP = Chloramphenicol, Amox = Amoxicillin
**p<0.05 when compared with control.*

DISCUSSION

The desired activity of an antibiotic is to kill or prevent the growth of offending pathogenic bacteria, but yet, these drugs may also impact the host in an injurious manner (Barnhill *et al*., 2012). Although there are generalized adverse effects common to most antibiotic,

for example gastrointestinal disorders, but certain antibiotics are associated with or manifest specific toxic effects. While some of these effects are mild, other consequence could be severe or even life threatening. These effects ranges from yellowing of teeth for tetracycline (Sánchez *et al*., 2004), reversible orange discoloration of skin and body fluids with rifampin (Holdiness, 1989) to ototoxicity and nephrotoxicity by aminoglycosides (Rougieret *et al*., 2004; Selimoglu, 2007), neuropathies and neoplasia associated with metronidazole (Hobson-Webb, 2006; Friedman *et al*., 2009), hepatitis caused by isoniazid and many others (Robles and Andrade, 2008; Sun *et al*., 2008). With evidence from varied reports, antibiotics have been considered as the largest group of agents that cause Drug-Induced Liver Injury (DILI) (Studniarz *et al*., 2012). These undesired effects have been largely blamed on the biotransformation of drugs to reactive intermediate metabolites or generation of reactive species and free radicals (Aruoma *et al*., 1989; Halliwell *et al*., 1992; Evans *et al*., 1994).

Chloramphenicol which was initially extracted from Streptomyces venezuelae is an antibiotic with a small molecular size and highly lipid soluble. Chloramphenicol has been indicated in wound healing, in the treatment of ocular infections, meningitis and various microorganism infections. It has been used as the last-line defense for multiple drug-resistant organisms like enterococcus (Hammett-Stabler and Johns, 1998). In spite of these beneficial effects, Chloramphenicol has been implicated in several toxic/adverse effects which include aplastic anemia, gray baby syndrome, and leukemogenesis (Robin *et al*., 1981; Hammett-Stabler and Johns, 1998; Holt *et al*., 1993). Recent studies have implicated Chloramphenicol in causing mitochondrial stress and damage, decreased ATP biosynthesis, induced matrix Metalloproteinase-13 expression, and Solid-Tumor Cell Invasion (Li *et al*., 2005; 2010) and also as a potent inhibitor of Cytochrome P450 Isoforms CYP2C19 and CYP3A4 in human liver microsomes (Park *et al*., 2003). Likewise, amoxicillin, a beta lactamic antibiotic which is widely used to treat infections in children has also been shown to exert some adverse effects like gastrointestinal irritation, diarrhoea (Ebadi, 1997; Martindale, 1996), nephrotoxicity (Tune and Hsu, 1995; Tune *et al*., 1996; Vijayalekshmy *et al*., 1992) and hepatotoxicity when co-administered with clavulanic acid (Rene *et al*., 1997; Fletcher *et al*., 1997; Deppermann *et al*., 1989).

Results obtained in this study suggest that administration of both Chloramphenicol and Amoxicillin induced membrane lipid peroxidation by significantly increasing TBARS and Diene conjugation, lead to depletion in microsomal protein content and inhibited membrane bound Ca^{2+}-ATPase and glucose-6-phosphatase activities. Chloramphenicol toxicity is believed to be mediated through the metabolism of the P- NO_2 group in its structure by nitro reduction process which leads to generation of reactive nitrogen species (Yunis, 1989). Chloramphenicol has been reported by various scientists to produce reactive nitrogen and oxygen species (RNS and ROS) (Agerbo *et al*., 1992; Matsuhashi *et al*., 1996; Miura *et al*., 1997; Karbowski *et al*., 1999). These species are highly reactive; attacking membranes, thiol groups and biomolecules, thus leading to their biodegradation, damage or dysfunction. In the same manner, Amox has a highly electronegative side chain that withdraws electrons from adjacent carbonyl group by inductive and mesomeric effects resulting in the formation of drug-induced radicals (Ajibola, 1989; Hewitt and Hammond, 1996). Also, the B-lactam ring on Amox structure could attack the thiol groups of membranes and enzymes.

Damage to polyunsaturated fatty acids tends to reduce membrane fluidity (Lutz *et al*., 1998) which is known to be essential for the integrity and proper functioning of biological membrane (Halliwell and Gutteridge, 1987), thereby altering both the physical properties of microsomes and intrinsic enzyme activities (Taratino *et al*., 2009). Results from this study showed an increase in TBARS, an indicator of lipid peroxidation and Diene conjugation, the first stage in the oxidation of arachidonic acid (Klein, 1970; Di Luzio, 1973) and onset of lipid peroxidation which may be the consequence of increment in the generation of reactive species in the animals treated with the drugs. This may also explain reasons for the inactivation of the membrane bound enzymes assayed for in this experiment. Total microsomal protein was found to be decreased in the drug-treated animals compared with the controls ($p<0.05$). This could be as a result of oxidation of amino acids especially by combined action of H_2O_2 and Fe^{2+} which may result in the formation of carbonyl derivative. Carbonyl modification of proteins has been shown to cause enzyme inactivation and likely enhance proteolysis (Sohal and Weindruich, 1996).

Ca^{2+}-ATPase and G-6-Pase have been shown to have free thiols that are in close proximity to unsaturated lipids (Graf *et al*. 1992). As such, they are suitable model targets for studying adverse effects of oxidative phenomena on membranes. This is because the thiol group of these enzymes are highly sensitive to oxidation and are highly reactive with oxidation products (Viner *et al*., 1997; Moreau *et al*., 1998). These two enzymes were adversely affected in this study. G-6-Pase is an enzyme that is mainly located in microsomes, whose activity is

modulated by covalent modification of important functional groups (e.g thiol) as well as changes in the surrounding phospholipid phase (Agerbo *et al*., 1992). The susceptibility of the thiol groups to attack by oxidants and β-lactam may explain why the activity of G-6-Pase was depleted in the drug-treated groups with amoxicillin group showing the greatest depletion, followed by Chloramphenicol -treated group. G-6-Pase is very sensitive to even small changes in its environment such as membrane perturbation and covalent binding, which plays a part in the toxic effect of some secondary lipid autoxidation product on the enzyme (Agerbo *et al*., 1992).

In the same manner, Ca^{2+}ATPase activity of the drug treated animals decreased significantly ($p<0.05$) compared to the control animals. The inhibition of Ca^{2+}ATPase in Chloramphenicol -treated animals could be due to the generation of ROS, RNS and nitroso radicals (R-NO-), the intermediate products of chloramphenicol metabolism which have been associated to its toxicity (Karbowski *et al*., 1999; Trepold *et al*., 2000). Ca^{2+}ATPase activity was also found to be decreased in the animals treated with amoxicillin. This could be explained according to the report of Topp and Christensen (1974) on the sensitivity of the β-lactam ring of penicillins to thiol attack which usually lead to irreversible formation of the covalent drug-enzyme complex. This may then result in damage or inhibition of enzymes or enzyme activities. Also, the highly electronegative side chain of amoxicillin that withdraws electrons from adjacent carbonyl groups resulting in radical formation could be held responsible for the decrease in Ca^{2+}ATPase activity of Amoxicillin-treated animals.

We could therefore conclude that treatment of rats for 10 days with the therapeutic doses of Chloramphenicol and Amoxicillin resulted in membrane lipid peroxidation, protein damage, and inhibition of microsomal Ca^{2+}ATPase and G-6-Pase due to increased generation of free radicals, reactive oxygen species and reactive nitrogen species. Effect of amoxicillin on the parameters in this study could also be due to the attack of its β-lactam ring on the thiol groups of the membrane and enzymes.

Evidences from this work could point to and support the contribution of amoxicillin in the hepatotoxicity of amoxicillin-clavulanic acid (Amoxiclav). There should therefore be moderation and great care in the use of antibiotics (drugs generally), while self-medication should be avoided totally. Likewise, certain antibiotics, especially in combined form should be reserved for the treatment of life threatening infections (a last line drug).

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