Histological and Biochemical Alterations on Oral Administration of Artesunate on the Testis of Male Mice

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ABSTRACT

Malaria is a global problem with the greatest burden of disease and mortality occurring in tropical and subtropical regions, especially in developing countries. The present study is focused to screen Artesunate toxicity in testis of male albino mice of Swiss Strain. Artesunate is a semi-synthetic water-soluble derivative of artemisinin generally used to treat chloroquine resistant malaria. Oral doses of 150mg/kg.b.wt and 300mg/kg.b.wt were administered for a period of 14, 21 and 45 days. Effects were observed on some specific parameters like 3β hydroxysteroid dehydrogenase, 17β hydroxysteroid dehydrogenase, cholesterol, protein and histology of concerned tissue using standard methods. The results of present investigation revealed that Artesunate caused significant alterations in a dose dependent manner. The histological findings after H&E staining indicated that the treated section of the testis showed some varying degree of cell clustering, cellular hypertrophy and intercellular vacuolations specifically in the germinal cell layer, resulting in a decline in sperm production. The germinal cell nuclei were highly pyknotic. Vacuolization within the interstitium was also observed particularly in the Leydig cell cytoplasm.

Keywords: Antimalarial Drug, Artesunate, Biochemical estimation, H&E staining, Testis, Toxicity.

INTRODUCTION

Malaria, a tropical disease caused by protozoan parasite of the genus *Plasmodium*, which has been a real concern for centuries and has now spread among 40% of the world’s population. *Plasmodium falciparum*, the most prevalent species across the globe, may cause cerebral malaria that is often fatal [1]. The treatment of malaria has posed great challenge to medicine and development of effective antimalarial drugs. This is due to
the development of resistance of parasite to most antimalarial agents [2, 3, 4] resulting in immense impact on the socio-economy of man [5, 6]. Resistance to chloroquine drugs is increasing, creating a need for new drugs that are well tolerated and simple to use. Hence, artesinin and its derivatives (artesunate, arteether, arteether, and dihydroartemisinin) have given renewed hope for combating resistant malaria [7, 8]. These drugs have gained considerable prominence in the chemotherapy of both uncomplicated and severe falciparum malaria by demonstrating high activity against multidrug-resistant falciparum strains with low toxicity profiles [9].

Artesunate is a drug used to treat malaria, especially chloroquine resistant malaria in Nigeria. It is a semisynthetic derivative of artesinin, the active component of the Chinese herb Artemisia annua, which consists of the sodium succinyl salt of dehydroartemisinin [10]. The choice of treatment for malaria has therefore metamorphosed from the inexpensive, effective and orally administered chloroquine to artesinin and its derivatives [11]. However, it has been reported that due to treatment failures associated with monotherapy of artesinins [12, 13], combined therapy of artesinin with other antimalarial agents known as the artesinin-based combination treatments (ACTs) have been recommended [14]. Artemisinin and its derivatives on their own have relatively low toxicological effects and that any toxicity observed in artesinin combination treatments may be due to the partner agents [15]. Several studies on artesunate showed evidence of toxicity on the brain stem [16, 17, and 18], superior colliculus [19], stomach [20] and testis [21]. With the increased efforts in the development of more potent anti-malaria agents as a result of the challenge posed by the resistant strains of the malaria parasite, the toxicity evaluation of these anti-malaria agents as their possible infertility actions becomes imperative. This is necessary since both malaria and infertility are Global problems that need to be focused on. The present investigation was therefore undertaken to evaluate the effect of oral administration of Artesunate on the reproductive tissue (testis) of male albino mice.

MATERIALS AND METHODS

Animals

Healthy, adult male albino mice (Mus musculus) of Swiss strain were used for the experiment. These mice weighed about 30-35 grams. The animals were housed in an air conditioned animal house at a temperature of 26 ± 2°C and exposed to 12h light: 12h dark cycle and were maintained on a standard Amrut mice feed (Pranav Agro Industries) and water ad libitum. Animals were caged separately in accordance with the experimental protocol in to different groups (Groups A to G) as per the guidelines set by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India and experimental protocols were approved by the institutional animals' ethics committee (167/1999/CPCSEA).

Experimental Design

Artesunate was prepared in double distilled water and orally given to mice via feeding canula with a hypodermic syringe. The two doses selected for the present study were based on therapeutic dose of artesunate administered for the treatment of uncomplicated malaria [22]. Adult Swiss albino male mice weighing between 30-35 g were kept in seven groups of ten animals each.

Experimental Protocol

Group A (control) received only feed and water ad libitum. Group B received Artesunate (150mg/kg.b.wt) for 14 days. Group C received Artesunate (150mg/kg.b.wt) for 21 days. Group D received Artesunate (150mg/kg.b.wt) for 45 days. Group E received Artesunate (300mg/kg.b.wt) for 14 days. Group F received Artesunate (300mg/kg.b.wt) for 21 days. Group G received Artesunate (300mg/kg.b.wt) for 45 days.

Artesunate was administered as per the experimental protocol. At the end of each treatment, animals were euthanized, dissected and testis was carefully dissected out, blotted free of blood and weighed. Tissue was then processed for biochemical and histological evaluation.

Protein estimation

Protein levels in the testis of control and all treated groups of animals were estimated by the method of Lowry [23]. Protein containing preparation when treated with phenol reagent of Folin-Ciocalteau, a deep blue colouration develops. This colour development is due to two reactions occurring simultaneously, i.e.
the reaction of alkaline copper sulphate solution with peptide bonds and the reduction of phosphomolybdic and phosphotungstic acids by aromatic amino acids present in the protein. The blue colour developed was quantitatively proportional to the total protein, which was measured colorimetrically at 540 nm.

**Cholesterol estimation**

The levels of cholesterol in the testis of control and all treated groups of mice were estimated by the method of Zlatkis [24]. In the presence of concentrated sulphuric acid and glacial acetic acid, cholesterol forms a coloured complex with ferric chloride (FeCl₃) which was measured on Systronics Digital Spectrophotometer 167 against blank.

**3β Hydroxysteroid dehydrogenase (3β HSD)**

The testicular 3β hydroxysteroid dehydrogenase (3β HSD) activity was assayed by the method of Talalay[25]. The enzyme 3β hydroxysteroid dehydrogenase acts on substrate 3β hydroxy 5β –androstane-17-one (epiandrosterone) and reduces nicotinamide adenine dinucleotide (NAD) and the absorbance was measured at 340 nm.

**17β Hydroxysteroid dehydrogenase (17β HSD)**

The testicular 17β hydroxysteroid dehydrogenase (17β HSD) activity was assayed by the method of Talalay [25]. The enzyme 17β hydroxysteroid dehydrogenase acts on substrate, testosterone and reduced nicotinamide adenine dinucleotide (NAD) to NADH and the absorbance was measured at 340 nm.

**Histological study**

Histological studies were carried out by using the standard technique of haematoxylene and eosin staining. For light microscopic examination testis tissues from each group were dissected out, blotted free of blood and fixed in 10% formalin immediately after the autopsy. Fixation was carried out at room temperature for 18 hours, after which they were transferred to 70% alcohol. Several changes of 70% alcohol were given, there after tissues were dehydrated by passing through ascending grades of alcohol, cleared in xylene, embedded in paraffin wax (58 to 60°C M.P) and transverse sections (T.S) were cut at 5µm on a rotary microtome. These sections were stained with haematoxylene and eosin, dehydrated, cleared in xylene and mounted in DPX (Distrene Plastisizer Xylene) as permanent slide. The photomicrographs of the relevant stained section slides were taken with the aid of a camera attached to biological light microscope.

**Statistics**

All the data are presented as MEAN±SE. Statistical analysis was performed using the trial version of SPSS software package version 16.0 (USA). Comparison between groups was made by one-way analysis of variance (ANOVA) taking significance at P < 0.05 followed by Student’s t-test taking significance at ***P < 0.001, **P < 0.005 and *P < 0.01. Tukey’s honestly significance difference (HSD) post hoc test was used for comparison among different treatment groups (P < 0.05).

**RESULTS**

**Terminal body weight and tissue weight**

Reduction in body weight (P<0.01) was observed in Artesunate 150 mg/kg bw treated mice for 21 and 45 days compared to control mice. While 7 and 14 days treatment showed insignificant reduction in terminal body weight as compared to control group. Maximum reduction, in body weight was observed in Artesunate 300 mg/kg body wt treated mice for duration of 45 days (Table 1).

Weight of testes declined following Artesunate treatment in a dose dependent manner. The reduction in tissue weight was highly significant (P<0.001) when 300 mg/kg bw of Artesunate was administered as compared with control mice (Table 1).

**Biochemical analysis**

Artesunate administration 300 mg/kg bw for 21 and 45 days significantly decreased the protein content in testis (P<0.001), while low dose of 150 mg/kg bw (14 and 21 days) did not show any significant changes and were found to be comparable with the control group values.

Oral administration of high dose of Artesunate for 45 days produced a significant increase (P<0.005) in cholesterol level in the testis when compared with the control group (Table 2). On the other hand low dose of (150 mg/kg bw) Artesunate for 14 days group-B and 21 days group-C treatment did not show any significant changes.
Activity of 3β HSD showed insignificant decrease in animals treated with 150mg/kg.b.wt of Artesunate for 14, 21 and 45 days. Where as it was significantly decreased (P<0.005) when 300mg/kg.b.wt high dose of artesunate was administered for 45 days. Similar pattern of results were also observed in 17β HSD activity (Table 3).

Histopathological analysis

Testis of control mouse showed well developed seminiferous tubules having basement membrane, germinal epithelial cells in different stages of division. The lumen contained bundles of sperms. Well developed Leydig cells were also observed (Fig. A, B).

Histological effects of Oral Administration of Artesunate on the testis of control and treated mice.

In the group of animals where Artesunate was administered at 150 mg/kg bw for 45 days histology of the testis was comparable to the testis of control mice. The nuclei of germinal epithelium cell were pyknotic and lumen contained few sperms and Leydig’s cells. No vacuolization was observed in the interstitium (Fig. C, D).

The histology of the testis of 45 days treated mice which were administered 300 mg/kg bw Artesunate revealed that the germinal layer was considerably disrupted and showed some varying degree of cell clustering.
Many tubules were almost devoid of germinal cell layer and sperm. Presence of giant cell was noted in the tubular lumen. The germinal cell nuclei were highly pyknotic. Vacuolization of interstitium was also observed. Leydig’s cells also appeared to have vacuoles in their cytoplasm (Fig. E, F).

DISCUSSION

Malaria is estimated to cause the death of over one million people annually, mainly children and pregnant women in Africa. Moreover, despite intensive research, the overall disease burden arising from infections by the malaria parasite Plasmodium falciparum is increasing. One major factor has been the emergence and spread of malaria parasites, which are resistant to antimalarial drugs such as chloroquine or pyrimethamine/sulphadoxine. Consequently, many countries have now introduced artemisinin (ART) derivatives as their first-line therapy, in combination with other drugs (such as mefloquine, amodiaquine, piperaquine, pyrimethamine/sulphadoxine or lumefantrine) [26].

Artemisinin-based combination therapies have recently been introduced in virtually all countries in which malaria is endemic, thereby making such drugs the most essential class of antimalarial agents. Artemisinin (qinghaosu) and its derivatives is a major advance in antimalarial treatment [27].

Artesunate, the most widely used artemisinin-related compounds, is a hemisuccinate derivative of dihydroartemisinin (DHA). It may be given parenterally, intravenously, intramuscularly, orally, or rectally. Oral artesunate is used either alone or in combination, usually with mefloquine [28].

The result of present investigation considering the use of Artesunate as first line of antimalarial drug in the current scenario, the present investigation was designed so as to evaluate the degree of reproductive damage that could possibly be caused due to the drug toxicity. Hence in the pilot study the testicular tissue was evaluated using two different ranges of the doses. Biochemically as well as histological studies were carried out.

The result of present investigation revealed that both the tissue weight and total body weight showed reduction in dose and time dependent pattern. Further, more the significant reductions were noted with higher dose administration and with lower dose the impact of drug toxicity was noted with increase in duration of treatment (Table 1).

In fact, it has been reported that an increase or decrease in relative or absolute weight of an organ after administering a chemical or drug is an indication of the toxic effect of that chemical [29, 30]. Also, the weight of male reproductive organs usually provides a useful reproductive risk assessment in experimental studies [29, 31] and testicular size is the best primary assessment for spermatogenesis, since the tubules and germinal elements account for approximately 98% of the testicular mass [32].

Furthermore, the lowering of total protein content observed in testicular tissue as well as total body weight of the artemisate treated animals indicate impairment on enzymes, receptors and glandular secretions. The decreased in protein synthesis with high dose of antimalarial drug chloroquine (CQ) has also been reported [33, 34]. The antimalarial therapeutic drugs probably block the enzymatic synthesis of DNA/RNA by forming complexes with DNA and preventing form acting as a template for its own replication or transcription of RNA [35].
Thus more often implicates the probable reason for lowering of spermatogenesis (Figure E) due to lack of availability of necessary proteins for cell division growth and differentiation of germ cells. Artemisinins have an effect on protein kinase C and its isoforms at the transductional level of cellular activity and calcium metabolism in smooth muscle activity [36].

Artesunate combination therapies could generate oxidative radical generation through impairment PKC (Protein kinase C) and Ca2+ metabolism, thus generating ROS (Reactive Oxygen Species) which in turn will cause cell damage [37].

In the present investigation the changes in reproductive (testis) tissue histology were observed by the sloughing of the germinal epithelium and reduction of in the population of germ cells. The changes were dose and time dependent. This implicates damage to the reproductive cells causing reduced sperm count leading to infertility. The observed increased/accumulation of cholesterol in testis (Table 2) also suggests that cholesterol metabolism, testicular androgenesis and consequent steroidogenesis are impaired post artemesunate treatment. Similar observations are also reported after chloroquine treatment [34]. Artesunate administration suppressed spermatogenesis [38]. Further, degenerative changes have also been reported to cause apoptosis and necrotic reduction in cell death [39, 40].

The activity of 3β HSD and 17β HSD also supports the observed impairment in steroidogenesis after antimalarial Artesunate treatment.

CONCLUSION

The pilot results of the present investigation suggest that Artesunate could be used as antimalarial drug, since the prolonged or long term use should be with adequate follow-up, particularly in developing countries where self-medication is especially common and sometimes at dosages higher than the therapeutic dose. Repeated doses without appropriate recommendation should be discouraged. Further, investigations are in progress to find an effective ameliorative agents to combat the drug toxicity and thereby preventing reproductive toxicity.

REFERENCES

Table 1: Body Weight and Organ Weight of control and treated animals.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Body weight (gram)</th>
<th>Testis (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Control</td>
<td>40.62 ± 0.57</td>
<td>121±1.20</td>
</tr>
<tr>
<td>B</td>
<td>Artesunate (150mg/kg.b.wt) for 14 days.</td>
<td>40.15± 0.38 NS</td>
<td>117±1.32 NS</td>
</tr>
<tr>
<td>C</td>
<td>Artesunate (150mg/kg.b.wt) for 21 days.</td>
<td>35.44 ± 0.51*</td>
<td>113± 0.98 NS</td>
</tr>
<tr>
<td>D</td>
<td>Artesunate (150mg/kg.b.wt) for 45 days.</td>
<td>33.42± 0.29*</td>
<td>110± 0.93*</td>
</tr>
<tr>
<td>E</td>
<td>Artesunate (300mg/kg.b.wt) for 14 days.</td>
<td>37.49 ± 0.54NS</td>
<td>108±1.26 NS</td>
</tr>
<tr>
<td>F</td>
<td>Artesunate (300mg/kg.b.wt) for 21 days.</td>
<td>34.27± 0.49 *</td>
<td>103±1.32 **</td>
</tr>
<tr>
<td>G</td>
<td>Artesunate (300mg/kg.b.wt) for 45 days.</td>
<td>30.87 ± 0.25***</td>
<td>98 ± 1.23 ***</td>
</tr>
</tbody>
</table>
Values are mean ± S.E., *p<0.01, **p<0.005, ***p<0.001, NS-Non Significant
Analysis of variance at p < 0.05 significance level.

Table 2: Protein and Cholesterol content in testis of control and treated animals.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Protein</th>
<th>Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Control</td>
<td>11.65 ±1.09</td>
<td>0.46 ±0.07</td>
</tr>
<tr>
<td>B</td>
<td>Artesunate (150mg/kg.b.wt) for 14 days.</td>
<td>11.64 ± 0.38 NS</td>
<td>0.48 ±0.04 NS</td>
</tr>
<tr>
<td>C</td>
<td>Artesunate (150mg/kg.b.wt) for 21 days.</td>
<td>11.62 ± 0.51 NS</td>
<td>0.51 ± 0.08 NS</td>
</tr>
<tr>
<td>D</td>
<td>Artesunate (150mg/kg.b.wt) for 45 days.</td>
<td>10.45 ± 0.29 **</td>
<td>0.52 ± 0.02 NS</td>
</tr>
<tr>
<td>E</td>
<td>Artesunate (300mg/kg.b.wt) for 14 days.</td>
<td>11.19 ± 0.54 NS</td>
<td>0.56 ± 0.07 NS</td>
</tr>
<tr>
<td>F</td>
<td>Artesunate (300mg/kg.b.wt) for 21 days.</td>
<td>10.42 ± 0.49 **</td>
<td>0.65 ± 0.04 **</td>
</tr>
<tr>
<td>G</td>
<td>Artesunate (300mg/kg.b.wt) for 45 days.</td>
<td>8.65 ± 0.25 ***</td>
<td>0.73 ± 0.02 **</td>
</tr>
</tbody>
</table>

Values are mean ± S.E., *p<0.01, **p<0.005, ***p<0.001, NS-Non Significant
Analysis of variance at p < 0.05 significance level.
Table 3: Showing alteration in 3β HSD and 17β HSD content in testis of control and treated animals.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>3β HSD</th>
<th>17β HSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Control</td>
<td>0.288 ± 0.007</td>
<td>0.208 ± 0.006</td>
</tr>
<tr>
<td>B</td>
<td>Artesunate (150mg/kg b.wt) for 14 days</td>
<td>0.285 ± 0.004&lt;sup&gt;NS&lt;/sup&gt;</td>
<td>0.199 ± 0.003&lt;sup&gt;NS&lt;/sup&gt;</td>
</tr>
<tr>
<td>C</td>
<td>Artesunate (150mg/kg b.wt) for 21 days</td>
<td>0.289 ± 0.009&lt;sup&gt;NS&lt;/sup&gt;</td>
<td>0.196 ± 0.008&lt;sup&gt;NS&lt;/sup&gt;</td>
</tr>
<tr>
<td>D</td>
<td>Artesunate (150mg/kg b.wt) for 45 days</td>
<td>0.264 ± 0.006&lt;sup&gt;NS&lt;/sup&gt;</td>
<td>0.175 ± 0.003*</td>
</tr>
<tr>
<td>E</td>
<td>Artesunate (300mg/kg b.wt) for 14 days</td>
<td>0.272 ± 0.002&lt;sup&gt;NS&lt;/sup&gt;</td>
<td>0.202 ± 0.007&lt;sup&gt;NS&lt;/sup&gt;</td>
</tr>
<tr>
<td>F</td>
<td>Artesunate (300mg/kg b.wt) for 21 days</td>
<td>0.250 ± 0.005 *</td>
<td>0.164 ± 0.004 *</td>
</tr>
<tr>
<td>G</td>
<td>Artesunate (300mg/kg b.wt) for 45 days</td>
<td>0.236 ± 0.008**</td>
<td>0.145 ± 0.002**</td>
</tr>
</tbody>
</table>

Values are mean ± S.E., *p<0.01, **p<0.005, *** p<0.001, NS-Non Significant Analysis of variance at p < 0.05 significance level.