RESEARCH ARTICLE

The Cytogenetic Effect of Copper in Experimental Hypocupremic Goats

Mahmoud A. Allaam¹, Mohamed A. Nayel², Hany. G. Keshta³, Ahmed A. Zaghawa³, Ahmed M. elsify³, Akram A. Salama² and Hala A. Abou-Zeina³

¹. Department of animal medicine and infectious diseases.(Animal Medicine) Faculty of veterinary medicine-Sadat city university, Sadat city, Egypt  
². Department of animal medicine and infectious diseases.(infectious diseases) Faculty of veterinary medicine-Sadat city university, Sadat city, Egypt  
³. Department of Parasitology and Animal Diseases. Veterinary Research Division, National Research Center, Dokki, Egypt.

Abstract

The present experiment was conducted to investigate the effects of copper deficiency in goats on, DNA and chromosomes aberrations. A total number of 16 male goats of 12 months to 1.5 years old were used in the present experiment for 24 weeks. The male goats were randomly divided into two groups. The first group consisted of six animals and was kept as control fed copper sufficient diet. The second group was consisted of ten male goats fed diet treated with molybdenum and sulphur for 24 weeks. Blood samples were collected from all experimental animals every 6 weeks for 24 weeks with heparin for chromosomal analysis and comet assay. The clinical signs were recorded throughout the experimental period. Clinical examination of hypocupremic goats showed changes in hair color and texture at the 9th week of the experiment. The copper deficient animals were depressed, listless, nervous (hyperesthesia), anemic (exhibited pale mucus membranes) and diaphoric in some goats, as opposed to control group. There was marked increase in comet numbers in Cu deficient group in all animals compared with the same group at zero time. And there was high significant increase in chromosomal aberration at the end of the experiment in which there was increase in structural abnormalities as break, deletion, fragment and centric fusion which were high significant compared to the analysis at zero time. Moreover, there was significant increase in numerical abnormalities as hypoploidy and polyploidy which was significant increase in number after induction of Cu deficiency compared to analysis at zero time.

Thus, the plan of the present work was designed to fulfill the following points:-
1- Assessment of DNA damage induced by Cu deficiency by comet assay.
2- Assessment of structural chromosomal aberrations induced by Cu deficiency by cytogenetic analysis.

1. Introduction.
The role of trace minerals in animal production is an area of strong interest for producers, feed manufactures, veterinarians and scientists. Adequate trace mineral intake and absorption is required for a variety of metabolic functions including immune response to pathogenic challenge, reproduction and growth (Larson, 2005). The physiological role of Cu in the body is related to several functions, which include cellular respiration, bone formation, connective tissue development and essential catalytic cofactor of some metalloenzymes (Vazquez-Armijo et al., 2011). Cu is required for the activity of enzymes associated with ferrous metabolism, elastin and collagen formation, melanin production and integrity of central nervous system (Abd El-Raof and Ghanem, 2006).

Hypocupremia in cattle is associated with an increase in the frequency of chromosomal aberrations as well as in DNA migration as assessed by the comet assay. Whereas the comet assay could differentiate Cu plasma level groups, chromosomal aberrations only detected differences between normal and hypocupremic animals. The increase of DNA damage found in hypocupremic animals could be explained by higher oxidative stress suffered by these animals (Piccoet al., 2004). The effect of the Cu deficiency on the induction DNA damage through an increase of the oxidative stress has been proposed (Ceroneet al., 1998 and Keen et al., 1998). Copper plays an important supporting role in preventing oxidative damage to critical cellular components. It is often assumed that failure to provide cells and tissues with an adequate supply of Cu, increase their susceptibility to oxidative stress. Moreover, normal levels of Cu must be present in cells to maintain the structural integrity of DNA during oxidative stress. Therefore, Cu deficiency promotes oxidative DNA damage (Pan and Loo, 2000). The increase of chromosomal damage in hypocupremic cattle was described and the frequency of chromosomal aberrations (mainly chromatid and chromosome breaks) was significantly higher than that found in normocupremic animals. The Cu level in plasma is inversely correlated with DNA damage. (Abba et al., 2000).

The structural integrity of the nuclear matrix associated with chromosomal DNA indicated that Cu ions are important for maintaining at least one level of folding of the DNA strands. Moreover, it is clear that nuclei contain a significant proportion of cellular Cu and that much of that is actually bound to DNA bases (Linder 2012).

2. Material and Methods:

2.1. Experimental animals:

A total number of sixteen clinically healthy adult castrated baladi male goats their ages 1 - 1.5 years old and weighted 19±0.82 kg were used in the present experiment. The experimental animals were dewarmed with systemic anthelmentic (Ivermectin + Clorsulon 1 ml / 50 kg Bwt, Ivomec super R manufactured by Merial Saude Animal Ltda) and were exposed to the periodical fecal examination all the time of the experiment (24 weeks). The animals were placed in good hygienically well ventilated stable and kept under the same environmental, nutritional and hygienic conditions throughout the period of the experiment. The animals were left for 2 weeks for acclimatization before the beginning of the experiment. Goats were subjected to periodic clinical and laboratory examinations and were apparently healthy at the time of the experiment.

2.2. Experimental Diet:

The ration offered to the animal was basically composed of: 50% yellow corn, 25% cotton seed cake and 17% wheat bran (Table 1). Additionally, animals were supplemented with seasonal green fodders essentially Trifoliumalexandrinum (green barseem) in winter and sweet corn (green maize) in summer. However, roughages (wheat straw and rice straw) were added at night. Fresh drinking water was offered ad libitum. The ration offered to the animals all the period of the experiment were biochemically analyzed for detailed ingredients percentage as well as some trace elements contents.

2.3. General layout of the experiment:

The castrated male goats were randomly divided into two groups. Each group was placed in a separate pen. The first group (control) consisted of 6 animals and was kept as control fed Cu sufficient diet. The second group (hypocupremic goats) included 10 animals that were subjected to experimental induction of secondary Cu deficiency by gradual addition of Molybdenum (Mo) and Sulphur (S) for 24 weeks.

2.4. Chemical analysis of experimental diet:

Feed sample of experimental basal diet was collected and chemically analysed for detailed ingredients and nutritive values according to the techniques carried out by Association of Official Analysis Chemists (1990). Concentrations of N, P, K, Mg, Ca and Na were calculated as %; whereas, total contents of Fe, Mn, Zn and Cu were calculated as (ppm).

2.5. Induction of Cu deficiency:

Experimental induction of Cu deficiency was carried out by adding molybdenum and sulphur to the ration according to (Moenei et al., 2008) with some relevant modifications, as shown in table (2).

2.6. Clinical examination of experimental animals:
Thoroughly clinical examinations were conducted according to Radostitis et al., (2007). Parasitological examination was made according to BassertMcCurnin (2010). Blood samples were collected via jugular vein puncture at zero day, 6 weeks, 12 weeks, 18 weeks and 24 weeks of the experiment according to Radostitis et al. (2007).

The blood samples were collected in one vacutainer tube with heparin for blood lymphocyte culture for chromosomal analysis and comet assay. Erythrocytes lysate were obtained following centrifugation of the blood samples at 3000 rpm for 10 minutes for separation of plasma. The collected erythrocyte were lysed in 4 times its volume of ice cold HPLC grade water (deionized water) and centrifuged at 10,000 rpm for 10 minutes at 4 °C. Erythrocytes lysate was then collected and stored at -80 °C till analysis according to Nishikimi et al., (1972).

2.7. Assays:
2.7.1. Single Cell Gel Electrophoresis (SCGE) (Comet assay):
The Single Cell Gel Electrophoresis assay (also known as comet assay) is an uncomplicated and sensitive technique for the detection of DNA damage at the level of the individual eukaryotic cell. It has since increased in popularity as a standard technique for evaluation of DNA damage/repair, biomonitoring and genotoxicity testing. Comet assay was done according to modifications of Singh et al., 1988 and Klaude et al., 1996.

2.7.2. Metaphase chromosome preparation from cultured peripheral blood cells:
It occurs according to the method described by Hartmann and Speit, 1995. And occurs as follows:
- All incubations are performed in a humidified 37°C, 5% CO2 incubator unless otherwise specified.
- Collection of sample and initiate cultures
  1. Peripheral blood was collected by venipuncture into a sodium heparin Vacutainer or a syringe with 25 U preservative-free sodium heparin per milliliter of blood.
  2. 0.2 ml whole blood was added to 5 ml of lymphocyte culture medium.
  3. Incubate for 72 h at 37 °C.
  4. Metaphases were obtained by adding 0.2 µg/ml colchicines to the cultures 3 hrs before harvesting.
  5. Centrifuge 10 min at room temperature. Discard supernatant.
  6. 6 ml of hypotonic solution (0.075 M KCl) was added at room temperature and gently resuspend cells. Let stand 15 min at room temperature.
  7. Freshly made fixative was added dropwise with a Pasteur pipet and mix well.
  8. Leave in fixative for 30 min at 4 °C. Centrifuge at 200 g for 5 min, and then wash in three to five changes of fixative before making slides.
  9. Wipe slides clean with absolute ethanol just before use.
  10. Place a drop of cell suspension on each slide and air dry. Monitor the quality of chromosome spreading under phase contrast. Chromosomes should be well spread without visible cytoplasm.
  11. The slides were analyzed at 1000 magnification using a light microscope.
  12. One hundred metaphases cells were screened per each individual. Cells with 60 chromosomes were scored for chromosomal analysis.

2.8. Statistical analysis procedures:
All data were subjected to statistical analysis including the calculation of the mean and standard error. Significance between data of treated groups was evaluated by Student T-test and F-test (One Way Anova) at levels p < 0.05 according to Petrie and Watson (1999) using SPSS version 10 computer program.

3. Results
Table (1): Constituents formula of concentrate feed mixture used in the experiment.

<table>
<thead>
<tr>
<th>Feed ingredients</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Yellow corn</td>
</tr>
<tr>
<td>2</td>
<td>Cotton seed cake</td>
</tr>
<tr>
<td>3</td>
<td>Wheat bran</td>
</tr>
<tr>
<td>4</td>
<td>Molace</td>
</tr>
<tr>
<td>5</td>
<td>Lime stone</td>
</tr>
<tr>
<td>6</td>
<td>Common salt (commercial sodium chloride)</td>
</tr>
</tbody>
</table>

Table (2): Minerals supplements adding to daily diet during the experiment.
3.1. Ration analysis:
Chemical analysis of the experimental ration revealed that the copper content of the ration was 8 ppm (Table 3).

3.2. Clinical examination of the experimental animals:
Clinical examination of hypocupermic goats showed changes in hair color and texture at the 9th week of the experiment then fine hair became limp, glossy and steely appearance as compared with control ones. Moreover, the black hair showed depigmentation and become easily to be detached. The clinical signs of depigmentation and hair changes became obvious at the end of the experiment. The copper deficient animals were depressed, listless, nervous (hyperesthesia), anemic (exhibited pale mucus membranes) and diaphoric in some goats, and as opposed to control group as shown in (Fig 1,2).

3.3. Cytogenetic essay in experimental animals:

a. Single Cell Gel Electrophoresis (Comet assay):

There was increase in comet numbers in Cu deficient group in all animals compared with the same group at zero time as shown in (Figure 3 A,B,C,D), (Fig 4)

b. Blood lymphocyte culture (chromosomal analysis):

There was high significant (p<0.01) increase in chromosomal aberration at the end of the experiment in which there was increase in structural abnormalities as break, deletion, fragment and centric fusion which were high significant compared to the analysis at zero time. Moreover, there was significant (p<0.05) increase in numerical abnormalities as hypoploidy and polyploidy which was significant increase in number after treatment compared to analysis at zero time as shown in (Table 4, Figure 5).
Fig. 2. Copper-deficient goat showed emaciation
FIG 3 (A). Blue arrows showing Comet assay at Zero day which show normal cell without DNA fragmentation. (B) Showing Comet assay at the mid of experiment (12 weeks) show minor DNA fragmentation. (C) Showing Comet assay at the end of experiment (24 weeks) show major DNA fragmentation. (D) Showing Comet assay at the end of experiment (24 weeks) show major DNA fragmentation.

Figure (4) Comet assay in goats fed on copper-deficient-diet in zero time as a control and at the end of the experimental period (24 weeks).
Fig. 5  **White arrows showing** chromosomal aberrations in goats. (A) Chromosomal aberrations *(Polyploidy)*. (B) Centric fusion (C) Deletion (D) Break.
Table (3): Chemical constituents of the experimental ration given to control and experimentally induced copper deficient goats for 24 week.

<table>
<thead>
<tr>
<th>Element</th>
<th>Ration content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total nitrogen</td>
<td>1.95%</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>0.30 %</td>
</tr>
<tr>
<td>Potassium</td>
<td>0.92%</td>
</tr>
<tr>
<td>Calcium</td>
<td>0.34%</td>
</tr>
<tr>
<td>Magnesium</td>
<td>0.27%</td>
</tr>
<tr>
<td>Sodium</td>
<td>0.54%</td>
</tr>
<tr>
<td>Iron</td>
<td>61ppm</td>
</tr>
<tr>
<td>Manganese</td>
<td>27ppm</td>
</tr>
<tr>
<td>Zinc</td>
<td>81ppm</td>
</tr>
<tr>
<td>Copper</td>
<td>8ppm</td>
</tr>
</tbody>
</table>

Table (4): Chromosomal aberrations in goats fed on copper-deficient-diet at zero time as a control compared with the same group at the end of the experimental period (24 weeks).

<table>
<thead>
<tr>
<th>Aberrations</th>
<th>Copper deficient as a Control at zero time</th>
<th>Copper deficient at the end of the experimental period</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal cells</td>
<td>32.10±1.11a</td>
<td>18.80±1.20b</td>
</tr>
<tr>
<td>Structural abnormalities</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deletion</td>
<td>4.80±0.93a</td>
<td>8.60±0.73b</td>
</tr>
<tr>
<td>Fragment</td>
<td>3.80±0.86a</td>
<td>5.50±1.24b</td>
</tr>
<tr>
<td>Centric fusion</td>
<td>3.70±0.48a</td>
<td>6.20±0.69b</td>
</tr>
<tr>
<td>Break</td>
<td>0.90±0.34a</td>
<td>1.50±0.50b</td>
</tr>
<tr>
<td>Total</td>
<td>13.20±1.84a</td>
<td>21.80±2.34b</td>
</tr>
<tr>
<td>Numerical abnormalities</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypoploidy</td>
<td>3.60±0.45a</td>
<td>5.70±0.77b</td>
</tr>
<tr>
<td>Polyploidy</td>
<td>1.10±0.31a</td>
<td>3.70±1.11b</td>
</tr>
<tr>
<td>Total</td>
<td>4.70±0.61a</td>
<td>9.40±1.75b</td>
</tr>
</tbody>
</table>

Data are presented as mean± SE (SE = Standard error).

Mean values with different superscript letters are significantly different at p<0.05 within the same column.

**Discussion**

Little is known about Cu deficiency in goats (Robert, 2004). Therefore, the present study aimed to investigate the effect of the experimental induced Cu deficiency on the development of clinical signs on goats and assessment of DNA damage and chromosomal aberrations in blood of Cu-deficient animals. Analysis of concentrate feed mixture as shown in table (3) used in this experiment revealed that the Cu content of the ration was 8 ppm which is considered being sufficient for growth and production of goat according to National Research Council (2007) which cited that Cu requirements for goats were established at 8 to 10 ppm.

The experimental ration of the goat succeeds in induction of secondary Cu deficiency in goat in which we added 10-40 mg Mo/kg DM and 1.5 -3 gm S/kg DM to which coincided with the result obtained by Suttle (1991); Cerone et al. (1995); Moineet al. (2008) and Mubarak (1998) who added 10 mg Mo and 3 gm S/kg DM basis to the compound ration of lambs for induction of secondary Cu deficiency. The specific effect of Mo in producing clinical Cu deficiency symptoms can be detected when Mo combined with S in the rumen to form thiomolybdates which bind with high affinity to dietary Cu in addition to antagonize Cu metabolism by decreasing absorption, increasing biliary excretion of Cu and chelating Cu from metalloenzymes (Fry, 2011). The clinical signs
of secondary Cu deficiency are likely to be from formation of thiomolybdate (MoS₂) in the body (Sanjabet et al. 2003).

The clinical examination of Cu deficient goats showed changes in hair color and texture at 9th week of the experiment which became limp, glossy and steely appearance compared with apparently healthy control goats. Moreover, the black hair showed depigmentation and became easily detached. The clinical signs of hair changes and depigmentation became more obvious at the end of the experiment. These findings are in concurrence with findings of Maas and Bradford (1990) in ruminants; Mubarak (1998) and Salehet al. (1998) in sheep; Woodbury et al. (1999) in calves; Tiffany et al. (2002) in beef cattle; Abd El Raof and Ghanem (2006) in sheep and Soetanet et al. (2010) in domestic animals. Fry (2011) attributed hair depigmentation associated with Cu deficiency in cattle to reduction in the activity of tyrosinase which is Cu-dependent enzyme required for melanin synthesis.

The Cu deficient goats showed paleness of the conjunctival mucous membrane from the beginning of the 18th week of the experiment which considered as a sign of anemia. This result was similar to that obtained by Mubarak (1998); Sharma et al. (2005); El-Khawas etc. (2007); Hansen et al. (2009) and Soetanet al., (2010). Cu deficiency has been found to cause anemia due to disturbances in iron metabolism resulting in sequestration of iron by the liver due to decreased plasma CP activity which involved in the metabolism of tissue iron (Stable et al. 1993).

The animals fed on the experimental diet showed hyperthermia and nervous manifestation at the beginning of the 21st week of the experiment. These results coincided with those obtained by Maas and Bradford (1990), Cerone (1999) and Aupperle et al. (2001) who explained that the defects which affect the skeleton of Cu-deficient animals are biochemically related to disorder cross-linking of connective tissue proteins caused by a deficiency of lysyl oxidase. Disorders of nervous system have been linked to a lack of cuproenzymes dopamine-β-hydroxylase involved in conversion of dopamine to norepinephrine (Cerone etc., 2000a). Soetanet al. (2010) explained that Cu is necessary for formation of myelin sheath, thus Cu deficient animal exhibit nervous disorders.

Emaciation and loss of condition became more obvious signs on goats fed the experimental ration at the 24th week of experiment. These results are in agreement with those obtained by Maas and Bradford (1990), Cerone etc. (1998) and Hansen et al. (2009) in ruminants. The signs of growth retardation in Cu deficient animals may be attributed to reduction of the activity of Cu enzymes such as CCO which is important in energy production (National Research Council, 1996). In the late stages of Cu deficiency, the impairment of tissue oxidation causes interference with intermediary metabolism and loss of condition and failure of growth (Radoisticset al., 2007).

The most important aim of this work was to analyze chromosomal aberrations and DNA damage in goats due to Cu deficiency. Results obtained showed that hypocupremic animals at the end of experiment exhibited higher frequencies of chromosomal aberrations than the normocupremic goats (the same animals at zero time). Chromosomal aberrations include (structural abnormalities), as break, deletion, fragment and centric fusion, and (numerical abnormalities) as hypoploidy and polyploidy which were significant increase in number after experiment compared to analysis at zero time, this is in agreement with previous study of Abba et al., 2000. Besides, there was marked DNA damage in blood cells of the Cu deficient animals in which there was very high significant increase in comet numbers in Cu deficient group in all animals compared with the same group at zero time. Animals with DNA damage assessed by Comet assay. These results were similar to that obtained by Pan and Loo, 2000 and Picco et al., 2001.

The increase of the frequencies of chromosomal aberrations and DNA damage found in the hypocupremic group at the end of experiment could be explained by the higher oxidative stress suffered by these animals. This stress could originate through two main ways characteristic of animals suffering nutritional deficiencies. First, in animals with hypocuprosis a significant decrease of one of the main antioxidant enzymes, Cu/Zn-SOD, has been found (Boyne & Arthur, 1986; Babu& Failla, 1989; Suttle& Jones, 1986 and Hawk et al., 1995) simultaneously with a catalytic decrease of the respiratory chain enzyme cytochrome-c oxidase (Hawk et al., 1995; Boyne, 1978 and Davis & Mertz, 1987). Thus, partial oxygen reduction leading to an increase of free radicals together with an insufficient antioxidant activity of Cu/Zn-SOD could increase oxidative stress.

On the other hand, caeruloplasmin, main cupremic determinant, appears to be one of the most sensitive enzymes to Cu deficiency, being well known that low caeruloplasmin levels is related with an increased susceptibility to infections and tissue injuries. Reactive oxygen species, including the superoxide radical, hydrogen peroxide and the hydroxyl radical, can induce oxidative stress in vivo (Emerit, 1994; Mello Filho and Meneghin, 1984). It has been suggested that caeruloplasmin in plasma acts as an extracellular scavenger of free radicals and may thus protect cells against lipid peroxidation by superoxide and other radicals released from neutrophils and macrophages (Saenko et al., 1994 and Weiss, 1989). It is worth to mention that several noncuproenzymes such as catalases and glutathione peroxidase are known to be influenced by Cu deficiency. Together with SOD, these are the main antioxidant enzymes (Prohaska, 1990; Olin et al., 1994 and Lai et al., 1995). Therefore, the nuclear
components can be damaged by hydroxyl radicals generated by the presence of O$_2$ and H$_2$O$_2$. Second, a significant decrease in one of the main antioxidant enzymes, SOD has been found in animals suffering from hypocuprosis. Thus, partial oxygen reduction leading to an increase in free radicals and insufficient antioxidant activity of SOD could increase oxidative stress and consequently DNA damage (Piccoet al., 2004).

**Conclusion**

The results of this work concluded the followings:

1. Copper deficiency was successfully induced under experimental conditions in goats by adding of 10-40 mg molybdenum/kg DM and 1.5-3g Sulphur/kg DM gradually throughout 24 weeks.
2. Additions of molybdenum and sulphur to the dry ration for 24 weeks produced clinical, cytogenetic changes of copper deficiency.
3. Our findings suggest that that Cu deficiency has a significant role in induction of chromosomal aberrations, DNA damage. So Cu level should be strongly considered during formulation of rations in farm animals.

**References**


