THE EFFECT OF COPPER CHLORIDE UPON THE FOLLICLES STAGE IN MOUSE OVARIES
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Summary
Copper in excess is a potential teratogen in mice and chick embryos. There are only few experimental data regarding folliculogenesis in mouse ovaries. In order to obtain an insight into this aspect, the effect of CuCl₂ 70 mg% was investigated by microscopic evaluation of follicles at 2, 5 and 10 days after treatment. Data obtained showed that after 2 days of the treatment there are significantly more follicles in stage II, compared with the control group and with the others two experimental groups (5 and 10 days after treatment). Also, in the experimental group were recorded some atypical aspect of granulosa cells and oocyte itself. Data obtained confirm a harmful effect of copper upon folliculogenesis in the first 2 days after treatment, which disappear, gradually at 5 and 10 days after treatment.
Keywords: copper chloride, mice ovaries, folliculogenesis.

Introduction
Copper is an essential trace element as an integral component of many enzymes and proteins and is needed in a wide range of metabolic processes. (Georgeopoulos et al, 2001). Studies also showed that copper is required for infant growth, host defense mechanism, bone strength, red and white cell maturation, iron transport, cholesterol and glucose metabolism (Uauy et al, 1998). Copper can pass in the system circulation by absorption from the gastrointestinal tract, lungs and skin. Population may be exposed to copper at low concentration either voluntarily (by supplementation) or involuntary through intake of contaminate food and water (Rosmarie AF 1992).

Deficiency copper or copper in excess can affect the normal development of mouse, rat, hamster and chick embryos (Shepard and Lemire 2004).

Our experimental investigations showed the harmful effect of excess copper upon preimplantational mouse embryos in vivo and in vitro. 7mg/kg body weight CuCl₂ administration by gavage on day 1,2,3,4 of gestation was followed on day 4 late by no teratogen effects. In vitro, by adding 10 µmol/L CuCl₂ in culture medium, the developmental rhythm of embryos and the hatching of blastocysts were significantly affected (Checiu et al, 2001).

Administration of 70 mg% CuCl₂ to mouse female in day 9 of gestation showed at 48 and 72 hours developmental retardation, limbs anomalies and neural tube closure failure with exencephalia at a significantly high number of embryos. The results obtained in vivo were confirmed by those in vitro. In vitro effects were obtained by adding 10 mg and 17mg% CuCl₂ in culture medium which induced general embryos retardation (embryos were 9 days old and were cultivated for 48 hours). The retardation was expressed by a significantly
decrease of somite numbers and the crown-rump length. Also, neural tube closure defects at the anterior neuropore were recorded (Checiu et al., 2002-2003).

Another aspect which was investigated was the effect of 70 mg % CuCl₂ upon fetal mouse skeleton development. Copper chloride administrated on day 9 of gestation led at the control on day 19 to a decrease of fetal and placental weight. Evaluation of the fetal skeleton showed a marked teratogenic effect of copper upon some bones and ossification centers (Checiu et al., 2004).

50 mg % Cu Cl₂ administration at chick embryo into the subembryonic cavity at 30, 40, 48-50 hours of incubation induced a high mortality rate and 20 mg % Cu Cl₂ administration induced at 5 days after treatment a caudal syndrome-tailness and dysgenesys of the limbs. The microscopic examination of malformed embryos showed the following pathological changes: disorganization of the ventral part of caudal primordium with structural modifications of neural tube, paraxial mesoderm and caudal end of notochord (at 24 hours after treatment). At 48 hours after treatment, the caudal region was severely affected: complete disorganization of the caudal end of neural tube and notochord and necrosis in paraxial, somatic mesoderm. The electron microscopic investigations in this experiment, confirm the microscopic results. The ultrastructural modifications showed vacuolization of mitochondria in ecto-meso and endodermal cells and in neural tube, many residual bodies and necrotic cells (Checiu et al., 2002 - 2003).

The data presented show the harmful effect of copper in excess upon mouse and chick embryos and in this experimental investigation we tried to establish a potential effect of copper in excess upon folliculogenesis in mouse ovaries.

**Material and methods**

Experiments were performed on adult mice females of 25-30g body weight from Swiss strain. Animals were kept in following conditions: 12 hours light and 12 hours darkness; 21°C; food and water ad libitum. The experiment was carried out in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purpose. 30 females received CuCl₂ 70 mg% by intraperitoneal injection (i.p.) 0.1 ml/10 g body weight. The treated females were divided in three experimental groups, 10 females in each group. At 2, 5 and 10 days after treatment, the females were sacrificed by cervical dislocation and the ovaries were fixed in Bouin liquid, embedded in paraffin, sectioned at 8 microns and stained with hematoxylin-eosin. The females from control group - 10 females - were used as control group and their ovaries were prepared using the same technique as for the treated groups.

The examination of histological section were made at Olympus CKX41 microscope.

The developmental aspect of the follicles was recorded using a modified classification of Pedersen and Peters (1968) made by Sandor and Muresan (1995). The following stages were recorded and counted:

- **Stage I**- one layer of follicular cells. Fig.1
- **Stage II**- two-four layers of follicular cells. Fig. 2.
- **Stage III**- multilayered preantral follicle. Fig. 4.
Stage IV-onset of antrogenesis and medium developed antrum. Fig. 5.
Stage V-mature antral follicle. Fig. 6.

**Fig. 1.** Stage I – one layer of follicular cells. (400x)

**Fig. 2.** Stage II – 2 layers of follicular cells (400x).

**Fig. 3.** Stage II – 3 layers of follicular cells (400x).

**Fig. 4.** Stage III - multilayered preantral follicle (400x)

**Fig. 5.** Stage IV. Onset of antrogenesis. (400x)

**Fig. 6.** Stage V-mature antral follicle. (200x)
In order to avoid the repeated evaluation of the same follicle, the preantral follicles were counted on each 10\textsuperscript{th} histological section and the antral follicles were counted on each 20\textsuperscript{th} histological section. Data obtained after microscopic examination of the histological sections were statistically evaluated by $X^2$ test and the accepted level of significance was $p<0.05$.

Results and discussions

The microscopic examination of the histological slides allowed to evaluate the number of ovarian follicles, found in different stages of development at 2, 5 and 10 days after CuCl\textsubscript{2} administration. The slides obtained from the control group were also examined. The obtained data are summarized in table 1.

<table>
<thead>
<tr>
<th>Follicular stage /days after treatment</th>
<th>Day 2</th>
<th>Day 5</th>
<th>Day 10</th>
<th>Untreated group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>294/22%</td>
<td>302/23%</td>
<td>318/24%</td>
<td>322/24%</td>
</tr>
<tr>
<td>Stage II</td>
<td>387/29%*/**</td>
<td>302/23%**</td>
<td>305/23%**</td>
<td>281/21%**</td>
</tr>
<tr>
<td>Stage III</td>
<td>281/21%</td>
<td>289/22%</td>
<td>305/23%</td>
<td>308/23%</td>
</tr>
<tr>
<td>Stage IV</td>
<td>214/16%</td>
<td>236/18%</td>
<td>240/18%</td>
<td>241/18%</td>
</tr>
<tr>
<td>Stage V</td>
<td>160/12%</td>
<td>183/14%</td>
<td>159/12%</td>
<td>188/14%</td>
</tr>
<tr>
<td>Total number of follicles/percentage</td>
<td>1336/100%</td>
<td>1312/100%</td>
<td>1327/100%</td>
<td>1340/100%</td>
</tr>
</tbody>
</table>

* $p<0.001$; ** $p<0.01$

From the data presented in table 1 the following aspects are to be mentioneted:

- The preantral follicles percentage does not show significantly statistical differences between the treated and the control group. (72% at 2 days, 68% at 5 days and 70% at 10 days after treatment compared with 68% at the control group).
- The percentage of the follicles which started antrogenesis and the percentage of mature, antral follicles shows no significantly statistical differences between the treated and the control group (28% at 2 days, 32% at 5 days and 30% at 10 days after treatment compared with 32% at the control group).
- The only significantly statistical difference ($p<0.001$) recorded is the increase of the percentage of the follicles found in stage II of development in group, in day 2, compared with the control group. Also, the number of follicles in stage II at 2 days post treatment is significantly higher ($p<0.01$) compared with day 5 and 10 groups.

These results shows, a temporary slowing effect of folliculogenesis at early stages (passing from stage II at stage III) immediately after CuCl\textsubscript{2} administration. At 5 and 10 days after treatment this situation is not found.

The microscopic examination of the follicles from the 2 days treated group, in different developmental stages, showed some modifications, which are not founded in all other groups.

These modification are:

- oocytes with fragmentation of the cytoplasm (fig.7):
- modification of the granulosa cells architecture, by rarefaction (fig.8)
- apoptotic follicular cells and modified oocytes (fig.9).
- apoptotic cells around antrum and abnormal aspect of the corona radiata (fig.10)
- apoptotic cells around zona pellucida (fig.11)
- apoptotic cells in antrum and granulosa (fig.12)

Fig.7. Follicle stage II, fragmentation of the oocyte (400x).

Fig.8. Follicle stage IV, rarefaction in granulosa (200x).

Fig.9. Follicle stage III. Follicular cells in apoptosis (400x)

Fig. 10. (200x). apoptotic cells around antrum and abnormal aspect of the corona radiata (fig.10)

Fig. 11. Follicle stage III. Apoptotic cells around zona pellucida (400x).

Fig. 12. Follicle stage IV. Apoptotic cells in antrum (400x).

Similar data (ovarian follicleatresia with cell debris and inflammatory cells in the antral cavity) were reported by Sakhaee et al, 2011, after administration of copper sulfate by gavage 200mg/ kg during 35 days.
There is a few data concerning the possible mechanism of copper negative influence upon ovary.

The accumulation of the redox-active transition metals (ex. Cu, Fe) in different tissues can be cytotoxic because probably induced increasing free radical production (Sayre et al, 1999). This oxidative stress is possible to induce the damage in ovary. New data are necessary for clarified this aspects.

Conclusions

Administration CuCl$_2$ at mouse females followed by their control at 2 days post treatment showed a significantly high number of the follicles in stage II, compared with control group.

The microscopic control of ovaries showed modifications of follicular cells and oocytes in all folliculogenesis stages:
- apoptotic follicular cells were noticed in granulosa, antrum and around zona pellucida;
- the architecture of the granulosa cells is modified by rarefaction;
- oocytes showed fragmentation and contracted cytoplasm.

Our results show a transitory harmful effect of CuCl$_2$ administration upon the beginning of folliculogenesis in the first two days after treatment. At 5 and 10 days post treatment these modifications were not found.

References


