INFLUENCE OF HYDROCOLLOIDS OF Ag, Au, AND Ag/Cu ALLOY NANOPARTICLES ON THE INFLAMMATORY STATE AT TRANSCRIPTIONAL LEVEL

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Abstract

The objective of this investigation was to evaluate the pro- or anti-inflammatory properties of nanoparticles of Ag, Au, and Ag/Cu alloy by examining the expression of NF-κB mRNA. The experiment was performed in ovo, on the chicken embryos’ model. The nanoparticles had no effect on embryos’ survival; the embryos from all groups were properly developed, without any abnormalities. Contrary to Ag and Au, nanoparticles of Ag/Cu increased NF-κB mRNA expression in embryo liver, indicating a pro-inflammatory effect. After treatment with LPS there was a significant decrease in NF-κB mRNA expression in the liver of embryos treated with Ag, compared to the placebo, Au, and Ag/Cu groups, indicating that Ag nanoparticles act as a potential anti-inflammatory factor. The results indicate the lack of influence of Ag and Au nanoparticles on NF-κB mRNA expression in chicken embryo liver. Contrary to Ag and Au, nanoparticles of Ag/Cu alloy may be considered as a pro-inflammatory factor. Nanoparticles of Ag, but not Au and Ag/Cu, can prevent over-expression of NF-κB mRNA after LPS stimulation.

Key words: chicken embryo, nanoparticle, gene expression, NF-κB, inflammation, therapy.

In recent years, numerous studies have been focused on anti-inflammatory therapy and on molecules which could block pro-inflammatory pathways. Nanoparticles of Ag and Au are considered as anti-inflammatory agents or components of anti-inflammatory molecules (3, 17). Recently, Kemp et al. (8) demonstrated that gold- or silver-heparin nanoparticles exhibit local anti-inflammatory properties without changing the systemic homeostasis. Consequently, it seems to be very important to explain whether pure nanoparticles of noble metals can show pro-inflammatory or anti-inflammatory properties which would determine their further application.

The nuclear factor κB (NF-κB) is a transcriptional factor that plays a key role in activating a cascade of processes involved in the defence of the organism, including pro-inflammatory pathways. NF-κB is sequestered in the cytoplasm and bound by inhibitory proteins - members of the IκB family. Phosphorylation of IκB by IκB kinase-β leads to the activation of NF-κB and the release of P50 and P65 subunits, which move to the nucleus and bind with a consensus sequence of various genes involved mainly in the immune defence activities, and thus activates their transcription (4). However, over-expression of NF-κB may also lead to subclinical chronic inflammation, and then transcriptional activity of NF-κB can promote cancer genesis (20).

The objective of this investigation was to evaluate the pro- or anti-inflammatory properties of nanoparticles of Ag, Au, and Ag/Cu alloy by examining the expression of NF-κB mRNA. The experiment was performed in ovo, on the chicken embryos’ model.

Material and Methods

Hydrocolloids of nanoparticles. Hydrocolloids of Au, Ag, and Ag/Cu alloy (60:40%), obtained from Nano-Tech, Poland, were produced by the electric non-explosive patented method (Polish patent 380649) from high-purity metals (99.99%) and high-purity demineralised water. The shape and size of the nanoparticles were inspected by transmission electron microscope (TEM) (JEOL model JEM-2000EX),
Picture 1. Samples of Ag, Au, and Ag/Cu nanoparticles for TEM were prepared by placing droplets of hydrocolloids on copper, formvarized grid (Agar Scientific Ltd., U.K.). Immediately after drying the droplets in dry air, grids were inserted into TEM.

Animal model. Fertilised eggs (n=200, 56 ±2.2 g) from Ross Line 308 hens were obtained from the Dembowska hatchery, Poland, and stored for 4 d at 12°C. Then the eggs were weighed and randomly divided into five groups, each with 40 eggs: group I (control) – not treated, group II (placebo) – physiological saline, group III (Ag) – hydrocolloid of Ag nanoparticles, group IV (Au) – hydrocolloid of Au nanoparticles, group V (Ag/Cu) – hydrocolloid of Ag/Cu alloy. Experimental solutions were given in ovo by injection into albumen (at 2/3 of the eggs’ height from the blunt ends) using a sterile 1 ml tuberculin syringe. The eggs were injected with 0.3 ml of physiological saline in the placebo group and with 0.3 ml of colloidal Ag, Au, or Ag/Cu nanoparticles at a concentration of 50 ppm. The injection holes were sealed with hypoallergic tape, and the eggs were placed in the incubator and incubated at standard conditions (temperature 37.7°C, humidity 60%, turned once per hour during the first 18 d, and later at temperature 37°C and humidity 70%). After 18 d, half the eggs from each group were treated with lipopolysaccharide (LPS) from the cell wall of Escherichia coli strain 0111:B4 (Sigma-Aldrich). LPS (0.4 mg/egg) was injected into the egg through the air cell. Then the eggs were taken out from the incubator, opened, and embryos were immediately killed by decapitation. The embryos were weighed and evaluated using the Hamburger and Hamilton stages of chicken embryo development (6), and the detailed morphological evaluation and weight of dissected organs. Immediately after decapitation, the livers were frozen in liquid nitrogen and stored at -80°C for pending analyses.

Total RNA extraction and reverse transcription (RT-PCR). Total RNA from the liver was isolated using NucleoSpin RNA II (Macherey-Nagel, Germany) according to the manufacturer’s instructions. The quantity and quality of total RNA was estimated by Nanodrop (Nanodrop, USA) and Bioanalyzer (Agilent, USA). Isolated RNA samples were treated with RNase-free DNase (Promega, USA) to remove any possible contaminating genomic DNA and then dissolved in diethylpyrocarbonate-treated water. The reverse transcription PCR (RT-PCR) was performed using 1 µg of RNA, and 100 U M-MLV of reverse transcriptase (Promega, USA) The RT-PCR product was stored at -20 °C for further use.

Primers and real-time PCR quantification. Six housekeeping genes (ACTB, GAPDH, B2M, TBP, 18S, and 28S) frequently used as a reference in real-time PCR gene-expression experiments, were tested for expression stability under the experimental conditions as previously described by Lisowski et al. (10). Primers for reference genes, as well as for target gene (NF-κB), were designed using the Primer 5 software (Whitehead Institute/Massachusetts Institute of Technology, USA) and G. gallus GenBank sequences. Primers were designed to produce amplicons spanning two exons – 2 and 3. The PCR amplification was performed in two independent runs by the 7500 ABI PRISM apparatus (Applied Biosystems, USA) using 96-well optical plates with a SYBR GREEN PCR Master Mix technique (Applied Biosystems, USA).

Data processing and statistical analysis. Data from two runs were calibrated by calculating the average cycle threshold value (Ct) over the samples in each run and the results were calculated using the mathematical model for relative quantification in real-time RT-PCR described by Pfaffl (14). The relative expression ratio of a target gene was calculated based on real-time PCR efficiencies (E) and Ct values of the target gene in comparison with a normalisation factor (NF). For subsequent normalisation, NF was obtained from the geometric mean of the raw expression data of two most stably-expressed reference genes, GAPDH and ACTB. Real-time E of the one cycle in the exponential phase was calculated from the gradients in the 7500 Real Time PCR System software according to the equation: $E = 10^{\frac{-1}{\text{slope}}}$.

Results obtained in the experiment were analysed using two-factorial and mono-factorial analyses of variance - ANOVA - and the differences between groups were tested by the multiple-range Duncan test, using Statgraphics Plus 4.1. Differences at P<0.05 were considered significant.

Results

Microscopic images of nanoparticles confirmed that the size of nanoparticles was less than 100 nm, wide-ranging with polygonal and spherical shapes (Fig. 1).

Fig. 1. Visualisation of nanoparticles of Au (left), Ag (centre), and Ag/Cu alloy (right), TEM x250k.
Table 1
The mortality, body, and liver weights and abnormalities in chicken embryos from control and from groups treated with placebo, and Ag, Au, or Ag/Cu nanoparticles

<table>
<thead>
<tr>
<th>Groups</th>
<th>control</th>
<th>placebo</th>
<th>Ag</th>
<th>Au</th>
<th>Ag/Cu</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mortality (%)</td>
<td>22.86</td>
<td>25.00</td>
<td>20.58</td>
<td>23.53</td>
<td>21.86</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>43.91&lt;sup&gt;a&lt;/sup&gt;</td>
<td>48.68&lt;sup&gt;b&lt;/sup&gt;</td>
<td>46.76&lt;sup&gt;b&lt;/sup&gt;</td>
<td>48.62&lt;sup&gt;b&lt;/sup&gt;</td>
<td>45.48&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.182</td>
<td>0.0173</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>0.88</td>
<td>0.93</td>
<td>0.94</td>
<td>0.94</td>
<td>1.00</td>
<td>0.031</td>
<td>0.1207</td>
</tr>
<tr>
<td>Liver g/100g b.w.</td>
<td>1.89</td>
<td>1.93</td>
<td>2.05</td>
<td>2.07</td>
<td>2.13</td>
<td>0.075</td>
<td>0.1094</td>
</tr>
<tr>
<td>Abnormalities*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> values within rows with different superscripts are significantly different;

* According to Hamburger and Hamilton (6) stages of chicken embryo development.

Table 2
The expression of mRNA NF-kB in chicken embryos’ liver from groups treated with PBS (placebo), Ag, Au, or Ag/Cu nanoparticles without and after LPS stimulation

<table>
<thead>
<tr>
<th>Groups</th>
<th>control</th>
<th>placebo</th>
<th>Ag</th>
<th>Au</th>
<th>Ag/Cu</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-kB mRNA without LPS stimulation</td>
<td>1.241&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.862&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.853&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.190&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.200&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.3342</td>
<td>0.0428</td>
</tr>
<tr>
<td>NF-kB mRNA after LPS stimulation</td>
<td>2.621&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.631&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.943&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.247&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.235&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.3891</td>
<td>0.0042</td>
</tr>
<tr>
<td>ANOVA**</td>
<td>SEM</td>
<td>0.2806</td>
<td>0.4222</td>
<td>0.3383</td>
<td>0.4143</td>
<td>0.4143</td>
<td>0.0296</td>
</tr>
<tr>
<td>P</td>
<td>0.0059</td>
<td>0.0016</td>
<td>0.1751</td>
<td>0.0056</td>
<td>0.0017</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> values within rows with different superscripts are significantly different.

The present results did not show any effects of the injection of hydrocolloids of Ag, Au, and Ag/Cu nanoparticles on embryos’ survival. The embryos from all groups were properly developed, without any abnormalities (Table 1). Morphology and weight of the liver did not vary between groups. However, body weight of embryos from placebo, Ag, Au, and Ag/Cu groups was greater than that in the control group.

The influence of hydrocolloids of nanoparticles (Ag, Au, Ag/Cu) on the mRNA NF-κB expression in chicken-embryo liver, without and after LPS stimulation, was presented in Table 2. The injection of Ag/Cu colloids significantly increase mRNA NF-κB compared with placebo, Ag, and Au groups but not the control group. After LPS stimulation, the lowest mRNA NF-κB expression was in the liver of chickens treated with Ag nanoparticles, comparing to placebo, Au, and Ag/Cu groups. The interaction between stimulation with LPS and treatment with Ag, Au, and Ag/Cu nanoparticles was insignificant. Additionally, the effect of LPS on mRNA NF-κB was evaluated within each group. The expression of NF-κB increased significantly after LPS treatment in all groups, except the Ag group.

Discussion

Chicken embryos, being highly-organised living structures, seem to be a better model for estimating the effects of nanoparticles on the intact organism than isolated in vitro cell cultures. This, independent from external influence, fast developing and easy-to-maintain animal model is quite well known – described in detail in the standard of Hamburger and Hamilton (6) and used in physiological and toxicological experiments (21). In previous experiments, we indicated that hydrocolloids of Ag and Ag/Cu, and also Ag/Pd, did not influence the mortality, growth, or development of chicken embryos and quails (5, 15). We also did not observe any redox status or DNA oxidative status alterations (16). The presented results also did not show any harmful effects on embryos development; however, the body weight of embryos from the placebo, Ag, Au, and Ag/Cu groups was bigger than in the control group, probably due to the injection of an additional amount of physiological saline into the eggs.

The NF-κB expression was determined in the liver of chicken embryos to evaluate whether nanoparticles of Au, Ag, and Ag/Cu alloy could modify inflammation at transcriptional level (Table 2).

In the presented experiment, nanoparticles of Ag and Au (50 ppm) had no influence on NF-κB
activation in the liver, at least at the level of the gene transcription. The results are in line with Ven et al. (19), who demonstrated highly-germicidal but absolutely non-toxic properties of silver nanoparticles in experiments with human fibroblasts. Furthermore, Kim et al. (9) indicated the lack of genotoxic properties of Ag nanoparticles. Nevertheless, nanoparticles can penetrate an organism, accumulate within tissues and cells (7), and even can cross the blood-brain barrier and accumulate in the brain, probably being harmful, but not via inflammatory pathways (18). Contrary to Ag and Au, nanoparticles of Ag/Cu alloy may be considered as a pro-inflammatory factor, since they increased NF-κB mRNA in chicken embryo liver. However, a significant increase in NF-κB mRNA expression was noticed in comparison, not to the control but to the placebo, Ag, and Au groups. The obtained result may suggest pro-inflammatory activities of copper, as it has been shown for copper ions (2) and copper nanoparticles (13).

To examine whether nanoparticles can be considered as anti-inflammatory agents, chicken embryos treated with Au, Ag, Ag/Cu, and placebo were stimulated by LPS injection. The mRNA NF-κB expression was the lowest in the liver of chickens from Ag group in comparison with the placebo, Au, and Ag/Cu groups. This preliminary observation can indicate silver nanoparticles as a potential anti-inflammatory factor. This result was clearly seen when the expression of NFκB was analysed at mRNA level in each groups without and with LPS treatment. Only in animals injected with Ag nanoparticles, the inflammatory responses were not observed. Moreover, we also noticed that the physiological level of mRNA NF-κB in chicken embryos from Ag group did not differ from control, suggesting that Ag did not block IκB phosphorylation or DNA-NFκB binding in the organism during physiological homeostasis.

NFκB is activated by pathogenic bacteria or their products (LPS, endotoxins) affecting cell receptors, or by stimulating cells by cytokines, miogens, reactive oxygen species, and viruses. From the present preliminary experiment it is impossible to suggest any metabolic pathways; however, the explanation can be related to the unique ability of Ag to “carry” oxygen (11). The oxygen embedded in the topmost silver layer is strongly bonded to the metal (1), probably being able to slightly modify tissue environment without changing redox status. It has been shown (12) that inflammation leads to a drop in oxygen levels in inflamed tissues. Hypothetically, Ag nanoparticles, as a kind of an oxygen carrier, could modify the surroundings of the inflammation site.

The presented results indicate the lack of influence of Ag and Au nanoparticles on NFκB mRNA expression in chicken embryo liver. Contrary to Ag and Au, nanoparticles of Ag/Cu alloy may be considered as a pro-inflammatory factor. Nanoparticle of Ag, but not Au and Ag/Cu alloy, can prevent over-expression of NFκB after LPS stimulation.

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References