Preliminary investigations on the use of photodynamic inactivation to treat open wounds contaminated with *Staphylococcus* and *Pseudomonas* bacteria and clinical evaluation of pigs

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ABSTRACT
To demonstrate and apply an alternative method of antimicrobial therapy, the bacteria from open wounds were inactivated photodynamically in Large White growing pigs with average initial weight of 11 kg, housed for 21 days in a specially fitted area. The lesions induced to the pigs and inoculated with *Staphylococcus aureus*, *Staphylococcus hyicus* and *Pseudomonas aeruginosa*, bacteria, didn’t change feed intake, didn’t cause losses in the animal stock and didn’t affect animal health, thus their performance. The average feed intake of 1.234 kg/pig produced an average daily gain of 612 g/pig. Each animal was monitored for the body temperature and it was concluded that the infection occurred only locally, where the lesions were, and didn’t affect the general state of the animal. The state of the wounds was monitored at regular periods starting with their inoculation with bacterial cultures until healing, observing the gradual reduction of the wounds until disappearance.

Keywords: growing pigs, wounds, bacteria, infection, method, irradiation, treatment.

INTRODUCTION
The existence, spreading and replication of pathogens in nature, which are the primary source of infection for human and animal organisms, and the incorrect or prolonged administration of various antibiotics, caused the treatments to lose efficiency and the bacteria to increase their resistance to these medications. *Staphylococcus* and *Pseudomonas* are among the bacteria with the highest resistance to antibiotics.

The research was thus directed towards finding alternatives to antimicrobial therapy, which to be more efficient and more rapid, non-toxic and non-invasive,
whose repeated application doesn’t increase bacteria resistance. Presently, the use of sources of coherent radiations (laser) is one of the priority research directions worldwide, aiming to inactivate bacteria using their photodynamic effect.

Based on the knowledge on the bacteria species sensitive to the photodynamic action of the light radiations, the method of photodynamic inactivation was used on bacteria of *Staphylococcus* and *Pseudomonas* genera, in open wounds on pigs. The following issues were considered:

- the direct effect on the bacterial population;
- the type of photosensitive substance which accumulated selectively in the isolated types of bacteria;
- ways of preparing the photosensitizers, according to the way of preparation and administration;
- manner of substance administration;
- determination of the therapeutic concentration of the photosensitive substances until the exposure of the wound to the coherent light radiations;
- type of source of coherent light pulsing or continuous emission;
- parameters of the light radiations (wave length, energy, duration of the pulse, pulse frequency, exposure time, etc.);
- ways of evaluating the biological response and treatment monitoring.

The photodynamic inactivation presumes first the administration of photosensitive substances, FS gel (photosensitizer), methylene blue solution, methylene blue gel, TBO gel (toluidene blue), TBO solution, which located the bacteria, followed by the irradiation of the contaminated area with coherent light on the wave length corresponding to the maximum absorption of the photosensitive substance. The photosensitizer molecule absorbed the coherent light and shifted to an excited state in which it reacted with the environmental oxygen, producing the singlet oxygen which is strongly reactive and which interacted with numerous types of cell enzymes, suppressed glycolysis and bacterial respiration, inhibiting protein synthesis, produced alteration in the cellular DNA molecule which contributed to the degradation of the genetic information transcription, DNA molecule replication, producing a mutagen effect which may be lethal to the bacteria.

Photodynamic therapy is a novelty, significant, promising for the treatment of yet other disorders and the cancer. Some scientific investigations show that this therapy has a high potential in many areas, but there is a strong need of expansion and search of clinics wanting to use it (Alexandrova et al., 2004).

**MATERIAL AND METHODS**

The experiment used pigs reared in the experimental farm of INCDBNA – IBNA Balotești, Large White, male growing pigs, with an average initial weight of 11 kg, housed in a specially fitted area.
A week before the experiment, the pigs selected for the experiment, representative for the particular group, were transferred temporarily in individual cages to get accustomed with the new conditions. The cages were placed in the experimental room, under constant 22ºC, and the animals had free access to the feed sand water.

**Organisation of the experiment:**

1) Preparation of the animals for inflicting the wounds:
   - all pigs were individualized by ear tags and the number recorded;
   - the day before the experiment started, the working area was shaved without affecting the skin;
   - before inflicting the wounds, the local area was disinfected with medicinal spirit;

2) The wounds were performed carefully, to obtain wounds as uniform as possible; the wounds were infected immediately as they were inflicted;

3) Broth cultures of *Staphylococcus aureus*, *Staphylococcus hyicus* and *Pseudomonas aeruginosa* were applied on the fresh wounds and each wound was painted so the culture enters the fresh wounds. After the primary infection was finished, to make sure the infection catches, a sterile dressing was imbibed with these cultures and applied and sealed with leucoplast;

4) The following types of infected wounds resulted:
   - control (no photosensitizing solution used);
   - experimental, using photosensitizing solutions with the following concentrations:
     - with FS (photosensitizing) gel;
     - with methylene blue solution, $3.3 \times 10^{-3}$ M;
     - with methylene blue gel sol., $3.3 \times 10^{-3}$ M (50ml sol./100 g GEL);
     - with TBO (toluidene blue) solution $3.6 \times 10^{-3}$ M;
     - with (toluidene blue) gel, sol. $3.6 \times 10^{-3}$ M.

5) After 24 hours from seeding, the reflection spectrum of each wound was measured for the first time (a beam of light was sent to the wound, part was absorbed, part was scattered), and the photosensitizing substances were administered;
6) After the photosensitizing substances were administered, each wound was covered in a dressing for 30 minutes, which was the waiting time in darkness, before irradiation;

7) Each wound was irradiated for 15 minutes with UFESA equipment belonging to the National Research Development Institute for Optoelectronics INOE – 2002.

8) Each irradiation was followed by the measurement of body temperature (thermogram), and then the reflection spectrum was measured again.

These determinations proceeded for 21 days, time during which we also monitored:

- the average daily feed intake;
- body weight evolution by the individual weighing of the piglets at the beginning of each week and in the end of the experiment;
- animal health.

The nutritive value of the feed ingredients was determined before the compound feeds were manufactured, using regression equations, and the nutritive value of the forages was expressed in corrected metabolisable energy cME and digestible crude protein (DCP).

These evaluations were performed on the basis of the determinations of chemical composition of the forages, according to the new system of assessing the energy and protein value of the forages.
Table 1 shows the compound feed formulation given to the animals during the determinations, and it was tailored according to the feeding requirements for the specific weight group. We used the indicators of the new system of assessing the energy and protein value of the forages (Burlacu et al., 2002), the quality indices being correlated with the specific requirements of this category of pigs.

The animals had free access to the feed and water; feed intake and feed leftovers were recorded daily.

Table 1 Compound feed formulation and quality indices

<table>
<thead>
<tr>
<th>Feed ingredient</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>42.2</td>
</tr>
<tr>
<td>Barley</td>
<td>22.0</td>
</tr>
<tr>
<td>Canola meal</td>
<td>6.00</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>16.00</td>
</tr>
<tr>
<td>Gluten</td>
<td>2.00</td>
</tr>
<tr>
<td>Powder milk</td>
<td>5.00</td>
</tr>
<tr>
<td>Oil</td>
<td>2.00</td>
</tr>
<tr>
<td>Monocalcium phosphate</td>
<td>1.40</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>1.50</td>
</tr>
<tr>
<td>Salt</td>
<td>0.20</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.15</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.45</td>
</tr>
<tr>
<td>Choline</td>
<td>0.10</td>
</tr>
<tr>
<td>Vitamin-mineral premix</td>
<td>1.00</td>
</tr>
</tbody>
</table>

**Analysed**

<table>
<thead>
<tr>
<th>Analysed Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude protein (%)</td>
<td>18.35</td>
</tr>
<tr>
<td>Lysine (%)</td>
<td>1.22</td>
</tr>
<tr>
<td>Methionine +Cystine (%)</td>
<td>0.793</td>
</tr>
<tr>
<td>Calcium (%)</td>
<td>0.935</td>
</tr>
<tr>
<td>Phosphors (%)</td>
<td>0.70</td>
</tr>
<tr>
<td>Metabolisable energy (Kcal/Kg)</td>
<td>3239</td>
</tr>
</tbody>
</table>

**RESULTS AND DISCUSSION**

Table 2 shows the average feed intake and the evolution of the body weight. The experimental data were processed statistically (Sandu Gh., 1995).

Table 2 Animal performance

<table>
<thead>
<tr>
<th>Item</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound feed intake, kg/pig/day</td>
<td>1.234</td>
</tr>
<tr>
<td>Initial average weight, kg/pig</td>
<td>11.25±0.875</td>
</tr>
<tr>
<td>Final average weight, kg/pig</td>
<td>21.65±2.15</td>
</tr>
<tr>
<td>Total average gain, kg/pig</td>
<td>10.40</td>
</tr>
<tr>
<td>Average daily gain, g/pig/day</td>
<td>612</td>
</tr>
</tbody>
</table>
As the table shows, the average feed intake was 1.234 kg/pig, which produced an average daily weight gain of 612 g/pig.

The experimental infection of the wounds with *Staphylococcus aureus*, *Staphylococcus hyicus* and *Pseudomonas aeruginosa* bacteria didn’t influence feed intake, didn’t cause losses in the animal stock and didn’t affect animal health, thus their performance. The state of the wounds was monitored at regular periods starting with their inoculation with bacterial cultures until healing, observing the gradual reduction of the wounds until disappearance.

The size of the wounds decreased gradually, at 2/3 days intervals, from 2 cm to 1.7 cm, to 1.3 cm and to 0.5 cm and eventually disappeared altogether.

Each animal was monitored daily for its rectal temperature, which showed that the infectious state was contained at the infected site, where reactions to the wounds occurred; the general health state of the animal wasn’t affected, the body temperature averaging 39.5 to 40°C, which fall within the limits of this age group, i.e. 39 – 40.5°C (Adameșteanu et al., 1959; 1966).

The clinical evaluation of the animals was done after they were accustomed to the environment, with the animal handlers and with the diets.

In handling the pigs we took care to avoid stress and cause then trauma, using the best suited methods of contention and protection.

Animal handling presumed getting close to the animal so it becomes used to the presence of people.

We used the recommendations of Vlăgioiu C. et al., 2001, concerning experimental animals handling:

- the handler stood on the opposite side of the examiner, holding the animal in his arms;
- the examiner demanded information from the handler concerning the character of the animal (if it kicks);
- the animal was approached perpendicularly on the working area, from the caudo-cranial direction, avoiding thus the limbs of the pig, which it uses for defence and attack; the examiner alerted the pig on his intention to get closer, but made no sudden gestures.
We actually caught the animal from the tail or from the hind limb, above the knee, holding it firmly. During the approach, the examiner addressed the animals by different firm commands or calling its name.

Animal contention presumed their immobilization, holding and limiting their possibilities of defence by aggressiveness, allowing the examiner free access to the working area.

We used the recommendations of Popa V. V. et al., 1982, to immobilize the animals:

- scratch the animal at the base of the ears or on the side of the belly;
- hold the animal from its ears and tail;
- tie a cord on its snout, behind the fangs;
- get hold of the snout of back of the neck with a long arm tonsils;
- get hold of a pelvis member with a cord;
- put the animal into a narrow cage to limit its movements;
- hold the piglets in arms.

The animals were monitored permanently while performing these procedures, to avoid them kicking with their head or limbs.

Every day the animals were submitted to the same routine: inspection, palpation, percussion, auscultation and thermometry.

The inspection was done using the visual, olfactory and auditory organs, with attention and comparatively, in natural light, under specially arranged conditions throughout the experimental period.

The general inspection and the close inspection didn’t reveal drainage from the infected wounds or purine smell, which shows that there were proper zoohygienical conditions.

Palpation was done with gradual intensity, comparatively and symmetrically, with the tips of the fingers, with the full palm, which shouldn’t be cold, so as not to produce reflex muscular reactions which could be misinterpreted. Thus, besides the morphological qualities which were normal, the palpation also allowed to evaluate some functional alterations in the intensity and frequency of activity (heart, lung).

Percussion was done patiently, symmetrically and comparatively, detecting and interpreting the usual sounds: high or low (according to the intensity of the vibrations), strong or weak (according to their amplitude) long or short (according to their duration), musical or noisy (according to the timbre. Percussion was done comparatively to clarify the quality of the sound, in neighboring areas, symmetrical or normal.

Auscultation was done to determine the normal or pathological sounds produced by organs (heart, lung, intestine, stomach) during their activity and it was done directly by putting the ear on the animal, and indirectly with a stethoscope.

Thermometry was evaluated indirectly both on the basis of the anamnesis data and of the clinical signs specific to the fever syndrome; during the experiment no signs of shivering, horripilation, dry snout, constipation, oliguria
were noticed. We also used the maximal thermometer to determine the rectal temperature of the pigs. No abnormal body temperatures were noticed in relation to the general state of the animal.

The apparatuses, systems and organs were valuated clinically, particularly the cavity organs which can also be examined from the exterior.

Usually, the evaluation started with the target organ or apparatus, following compulsorily with the other apparatuses or systems, knowing the existence of interfunctional relations between them which may disturb the normal activity of the whole organism (Vlăgioiu C., 2001).

Before examining the apparatuses, systems and organs, the animals were checked for their general state which monitored:
- the present general state (habitus);
- skin semiology;
- semiology of the apparent mucous;
- semiology of the superficial lymph system.

The habitus concerns the general outer look of an animal and it includes the evaluation of the conformation, constitution, welfare, facies, temperament and attitudes of the animal. A wider evaluation of the habitus also included data on the skin, apparent mucous and superficial lymph system.

Even from the beginning of the experiment, we considered the recommendations concerning the technique for animal examination (Vlăgioiu C., 2001), regarding the prodrom or the prodromic state, which is the early stage in the clinical evolution of the disease, starting when the first signs of disease appear, until the specific symptoms of a disease are obvious. This period is characterised by general clinical signs, discrete, incomplete, dubious, unclear, non-characteristic, there not specific to a disease. Such observations on the evolution of the animal were performed continuously by the group of laboratory assistants responsible for the experiment. The expression “the animal doesn’t feel well” of the handler coincided with the latent period, of incubation or invasion observed in infectious diseases and it is between the moment the pathogen entered the organism until the first signs of disease appear (apparition of the prodromic state).

The pulse was determined by pressing an artery, feeling its deformation by the blood wave generated by the heart systole. The pulse was felt on the femoral artery on the inner side of the thigh and on the brachial artery and by heart auscultation.

The pulse was evaluated for the following characteristics:
- pulse frequency was identical to heart beat rate, 60 – 80 beats/minute;
- under normal physiological conditions the pulse was rhythmic;
- pulse duration was short;
- pulse amplitude or volume depended on the cardiac output and on the difference between the maximal and minimal tension of the
pulse, being evaluated by the amplitude of artery unevenness and it was not felt;
- blood pressure was evaluated indirectly by palpating an artery and it was very faint.

The breathing noise at close range showed:
- vocal manifestations which were normal without the change of tone and expression (more strident, fainter) etc.;
- panting had no pathologic significance;
- rattling breathing didn’t presume the presence of fluid secretions on the airways and in the mouth;
- sneezing was reflex due to nasal mucous irritation by different factors (rhinitis, dust).

CONCLUSIONS

The lesions induced to the pigs and inoculated with *Staphylococcus aureus*, *Staphylococcus hyicus* and *Pseudomonas aeruginosa*, bacteria, didn’t change feed intake, didn’t cause losses in the animal stock and didn’t affect animal health, thus their performance;

Each animal was monitored for the body temperature and it was concluded that the infection was only local, where reactions occurred to the wounds, the general state of the animal not being affected.

The state of the wounds was monitored at regular periods starting with their inoculation with bacterial cultures until healing, observing the gradual reduction of the wounds until disappearance.

REFERENCES