EFFECTS OF BENZIMIDAZOLES AS INHIBITORS OF THE METABOLISM OF WASHED PORCINE SPERMATOZOA

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In the bovine and some other species many of the problems of semen storage and handling have been solved. Practical long-term storage and handling techniques for boar semen, however, are in the developmental stages. By using metabolic inhibitors it may be possible to control or regulate metabolic pathways and perhaps improve present methods of storage. Blackwood and Harris (1960) and Harris, Wilcox and Shaffner (1961) were able to prolong the storage life of chicken semen by using benzimidazoles and then diluting them. Similar research has not been conducted on boar semen.

Experimental Procedure

Semen was collected from six mature boars using the technique described by Herrick and Self (1962). Pooled semen from six boars was used in order to insure sufficient numbers of washed spermatozoa. Following the removal of gelatinous material, the semen was centrifuged at 650 g for 6 min. at 37 C, and the supernatant was removed by aspiration. The spermatozoa were resuspended in a modified Illinois Variable Temperature Extender (table 1), (VanDemark and Sharma, 1957). Centrifugation and aspiration were repeated twice (temperature was kept at 37 C and cells resuspended in IVT). Sperm cell concentration was determined with a hemocytometer and sperm cells were resuspended to obtain approximately 2 x 10⁹ sperm per 3 ml for each Warburg flask. Flasks were maintained at 37 C in the waterbath. Oxygen consumption was measured by the indirect method of Warburg as described by Umbreit, Burris and Stauffer (1964). Motility of the spermatozoa was estimated between 0 and 100% in intervals of 10. Lactic acid was determined by the method of Barker and Summerson (1941) and pyruvate by the method of Friedemann and Haugen (1943). The pH was determined with a glass electrode. Determinations were made after 0, 0.5, 1.0, 1.5 and 2 hr. of incubation. Three substituted benzimidazoles, 2-heptyl-5-methylbenzimidazole (HMB), 2-ethyl-5-methyl-benzimidazole (EMB) and 2,5-dimethylbenzimidazole (DMB) were studied. The following six treatments were conducted for each inhibitor: control, 1 mg ATP, 100 mcg of inhibitor, 100 mcg of inhibitor + 1 mg ATP, 300 mcg of inhibitor, and 300 mcg of inhibitor + 1 mg ATP. Four replicates were conducted for each inhibitor. Sufficient glucose (4 mg) was added to support glycolysis for 2 x 10⁹ sperm for 2 hours. Data were tested for significance with an analysis of variance program on the IBM 7094 computer.

Results

pH. The 300 mcg DMB and EMB samples had significantly (P<.01) higher pH's at 60 and 90 min. when compared to controls but none of the inhibitors had a significant effect over 120 min. or across inhibitor concentrations.

Motility. Both levels of HMB and the 300 mcg levels of EMB and DMB decreased (P<.01) motility (see figure 1). Motility scores for the control samples decreased from 72 to 62% during the 2-hr. incubation period. By comparison, the decline in motility due to inhibitors was from 45 to 15% for 300 mcg HMB, 60 to 21% for 100 mcg HMB, 72 to 30% for 300 mcg EMB and from 67 to 32% for 300 mcg DMB. Later work by Witters (unpublished data) showed that motility inhibition could be reversed up to 90 min. by washing the incubated sperm in IVT.

Oxygen Consumption. Both EMB and DMB significantly increased (P<.01) oxygen consumption over the controls across time while HMB did not significantly influence it.
TABLE 1. COMPOSITION OF THE PURDUE EXTENDER

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Weight (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose</td>
<td>0.30</td>
</tr>
<tr>
<td>Trisodium citrate</td>
<td>2.00</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.04</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>0.21</td>
</tr>
<tr>
<td>Streptomycin sulfate</td>
<td>0.30</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Bring the above ingredients to volume in a 100 ml volumetric flask with distilled water.

(See figure 1). The mean of oxygen consumption was 9.3 mcl for 100 mcg DMB samples, 8.3 for 300 mcg DMB, 6.8 for 100 mcg EMB, 7.1 for 300 mcg EMB, 5.4 for 100 mcg HMB and 5.0 for 300 mcg HMB.

Adding ATP had no significant effect on oxygen consumption for samples treated with the three benzimidazoles for 2 hr. of incubation.

**Lactic Acid.** Samples incubated with HMB and EMB had significantly lower (P<.01) lactic acid concentration across time and concentration (see figure 1) while DMB had no significant effect. Samples containing 300 mcg HMB showed the greatest inhibitory effect on lactic acid concentration. The 300 mcg HMB treated samples show a significant (P<.01) time interaction over 120 min. of incubation. Control samples average 6.05 mcg more lactic acid than did 300 mcg samples (see figure 1). DMB treated samples showed less variation across time when compared to control than the other inhibitors. The addition of ATP had no significant effect on lactic acid accumulation in DMB, HMB or EMB treated samples.

**Pyruvic Acid.** Pyruvate acid level in controls changed from 0.55 mcg per 10^8 sperm at 0 time to 0.63 mcg after 120 minutes. From 0 to 2 hr. pyruvic acid in the 100 mcg HMB samples ranged from 0.69 to 1.06 mcg per 10^8 cells and the 300 mcg HMB treated samples ranged from 0.60 to 0.85 mcg respectively. Only HMB significantly (P<.01) increased pyruvate concentration during the incubation (see figure 1). Addition of ATP did not significantly influence pyruvate production for treated samples.

**Discussion**

Metabolic effects of substituted benzimidazoles have been investigated in chicken sperm (Blackwood and Harris, 1960), in bacteria (McNair, Rogers and Rose, 1958), in polio virus (Hollingshead and Smith, 1958) and in chicken embryos (Blackwood and Shorb, 1958; Blackwood, 1962). In all the organisms examined, substituted benzimidazoles have acted as metabolic inhibitors. The substituted groups as well as their position on the molecule were important for this effect (Blackwood, 1960; Tamm et al., 1953); substitution at the two and five positions was essential for maximum activity. Tamm and coworkers also found that by increasing the size of the side-chain from methyl to ethyl at the two positions an increased inhibition could be attained. (The number of substituted side-chains and their location seemed to be important). Blackwood and Harris (1960) reported a decreased metabolic inhibition in diluted fowl semen.

The mode of action of the substituted benzimidazoles is not well understood in any organism or cell; no work has been published with reference to effect of benzimidazoles on mammalian sperm, but Tamm et al. (1953) working with Lee polio virus has postulated that, since 5,6-dimethyl-benzimidazole is a structural moiety of vitamin B-12, some of the substituted forms may interfere with synthesis of nucleotides in formation of nucleic acids. Working on the effects of benzimidazoles on chicken embryos Blackwood (1962), postulated that EMB acted as an inhibitor of RNA synthesis. Later work on related compounds (Baltimore et al., 1963) showed that 2-(-Hydroxybenzyl)-benzimidazole suppressed the production of poliovirus RNA polymerase.

**Pyruvic Acid.** In this study boar semen pyruvic acid accumulated up to 90 min. (across treatments) and then declined. This may be due to rapid conversion of available substrate to pyruvic acid, then a reduction as soon as the substrate is lowered due to increased oxidation and movement through the respiratory pathways. Terner (1959) has investigated pyruvic acid concentration in semen and has documented the presence of a dismutation reaction in which two molecules of pyruvic acid interact so that one is oxidized to acetate and carbon dioxide while the other undergoes simultaneous reduction to lactic acid. This probably explains some reports of rapid disappearance of pyruvic acid (Terner, 1959).

**Lactic Acid.** The three benzimidazoles showed an inhibitory effect on lactic acid production in the following order HMB>EMB>DMB. Blackwood and Harris (1960) also reported this effect on lactic acid production by chicken sperm and referred to
Figure 1. Effects of 0, 100 and 300 micrograms of benzimidazoles on pyruvic acid and lactic acid production, oxygen consumption, sperm motility and pH of porcine sperm incubated from 0 to 120 minutes. Standard errors are shown by lines in columns.
the side chains as being important in the degree of inhibition. HMB has a seven carbon side chain (heptyl) at carbon number two position while EMB and DMB have an ethyl and methyl group at carbon two, respectively. Since HMB and EMB inhibit lactic acid production, this could indicate a block somewhere prior to lactic acid formation. The HMB treated samples were significantly higher than controls in pyruvic acid and significantly lower in lactic acid after 120 minutes. This would be expected if the metabolic block is between pyruvic acid and lactic acid. The relationship between the increase in pyruvic acid and the decrease in lactic acid was not significant for EMB or DMB. Lactic acid values for boar semen may vary from 5 mcg per 10^8 cells as measured by Aalbers, Mann and Polge (1961) to 35 mcg per 10^8 cells as measured by Shelby (1966). The values for this study ranged from 10 to 21 mcg per 10^8 cells during 120 min. of incubation.

**pH.** The pH range of boar semen was between 7.60 and 7.65 (Shelby, 1966). The pH range in this experiment was 7.50 to 8.5. The pH of DMB and EMB samples increased significantly more than controls. HMB samples showed no significant pH change but were lower in pH than samples containing the other inhibitors. The inhibitor stock solutions were at a pH of 6.5 and the Purdue Extender was at a pH of 8.0. As the lactic acid and pyruvic acid accumulated, a decreased pH would be expected. The reason for EMB and DMB samples becoming alkaline is not known. The CO₂ absorbent used in the Warburg flasks was not KOH but a buffered diethanolamine (Pardee, 1949) which should not have caused alkaline pH. Dimethylbenzimidazole generally is the weakest inhibitor of the three and HMB the strongest. Whether this was important in pH change is not known.

**Oxygen Consumption.** Treatment with EMB and DMB increased the oxygen consumption over 2 hr. compared to controls, with EMB having the greatest effect. Oxygen consumption for the first 30 min. of incubation was higher for all three inhibitors than for the controls. These data are similar to data obtained from oxidative uncoupling in aerobic respiration (Chance and Williams, 1956). Oxygen consumption for HMB-treated samples did not increase over the 2-hr. incubation period.

Added ATP had no significant effect on oxygen consumption. In some cases of oxidative uncoupling the addition of ATP will stimulate the respiratory activity of tissues over long periods of incubation. Since this did not occur, the proof for oxidative uncoupling is not complete.

**Summary**

Three side-chain substituted benzimidazoles, DMB, EMB and HMB were incubated with washed boar sperm. Of the three, only HMB treatments significantly (P<.01) increased the pyruvic acid production by sperm. Different rates of inhibition were noticed for the benzimidazoles on lactic acid production with HMB inhibiting the most and DMB the least. The pH of EMB and DMB treated samples increased significantly (P<.01) more than controls. HMB samples showed no significant pH change but were lower in pH than samples containing the other inhibitors. HMB and the 300 mcg levels of EMB and DMB significantly lowered (P<.01) motility of sperm over 2 hours. Samples treated with all three inhibitors had significantly (P<.01) higher oxygen consumption after 30 min. of incubation but only EMB and DMB treated samples had significantly higher oxygen for 2 hr. of incubation. The effects on oxygen consumption are similar to those of oxidative uncoupling but more tests should be run on ATP level to verify this. The metabolic mode of action of benzimidazoles is unknown at present. Studies with polio and influenza virus have shown that RNA synthesis is blocked but no comparable investigation has been made with spermatozoa.

**Literature Cited**


