An evaluation of the outcome of bull castration by intra-testicular injection of ethanol and calcium chloride

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SUMMARY

In this study, the effectiveness of intra-testicular ethanol and calcium chloride administrations was compared for chemical-castration in 12 mix-bred young bulls (12-15 months old and 250-400 kg). Ten ml of absolute ethanol or 10 ml of a 30 % calcium chloride solution were administered intra-testicularly in each testicle which were removed with the open surgical technique after 60 days for histopathologic evaluation. Testicular swelling was evident in both group bulls following injection and reached peak within 48 hours. While testicular volume decreased significantly in ethanol group after 3 weeks, no significant change occurred in calcium chloride group. The testicles underwent atrophy at the 60th day in ethanol group with no marked alteration in calcium chloride group. Semen characteristics of only 3 bulls in ethanol group were very poor. Though only 3 bulls were infertile in ethanol injected group, other bulls were fertile.

The method is readily applicable with no major harms and adverse effects. If 50 % rate is considered satisfactory it can be used then in large flocks, in particularly where no chance of operation exists.

Key words : Chemo sterilization, ethanol, calcium chloride, bull.

RESUME

Évaluation de méthodes de castration de taurillons par injection intra-testiculaire d’éthanol et de chlorure de calcium.

Dans cette étude, deux méthodes de stérilisation chimique basées respectivement sur l’administration intratesticulaire d’éthanol ou de chlorure de calcium ont été évaluées séparément sur 2 groupes de 6 taurillons âgés de 12 à 15 mois et pesant de 250 à 400 kg. Dix ml d’éthanol absolu ou 10 ml d’une solution à 30 % de chlorure de calcium ont été administrés dans chaque testicule. Les testicules ont été prélevés chirurgicalement 60 jours après l’administration pour une évaluation histopathologique. Un gonflement des testicules a été mis en évidence suite à l’administration des deux produits avec un effet maximal observé dans les 48 h suivant l’administration. Alors que le volume testiculaire a été réduit dans le groupe traité à l’éthanol, 3 semaines après l’administration, aucun changement n’a été observé dans le groupe traité au chorure de calcium. Les testicules sont devenus atrophiés 60 jours après l’administration d’éthanol alors qu’ils ne présentaient pas d’altération marquée dans le groupe traité au chlorure de calcium. Les caractéristiques du sperme de seulement 3 taurillons traités à l’éthanol ont été très mauvaises et associées à une infertilité. Les 3 autres taurillons traités à l’éthanol étaient fertiles.

En conclusion, la stérilisation chimique basée sur l’administration d’éthanol pourrait être applicable dans de grands élevages sans effets néfastes si un taux de 50 % de réussite est considéré satisfaisant en l’absence d’intervention possible.

Mots-clés : Stérilisation chimique, éthanol, chlorure de calcium, taurillon

Introduction

Chemo sterilization was experimented in males monkeys, hamsters, rabbits, rats and dogs by intratesticular injection of some agents such as ferric chloride [19], danazol [6], BCG [5], zinc tannate [8], glycerol [14,17,30], glucose, NaCl [15,25] dibromochloropropane [27], lactic acid [11], zinc arginine [9], sodium fluoride [29], formaline [1], calcium chloride [18,26], ethanol [3], and potassium permanganate plus glacial acetic [13]. In male ruminants, intratesticularly lactic acid [16] tannic acid and zinc sulphate [10] alpha-hydroxypropionic acid [4], formalin [21] Castrate-Quin 14 [28] have been used, but because of many complications following the use of these chemicals, an effective chemo sterilization method has yet to be established.

The purpose of the study was to determine the efficacy of intratesticular ethanol and calcium chloride injections for chemo sterilization in bulls.

Materials and methods

In this study, 12 mix-breed young bulls (250-400 kg, 12-15 months old) were used. They were randomly divided into two groups, each group containing 6 bulls, and called EG (ethanol group) and CCG (calcium chloride group). Each intratesticular injection was performed using a sterile 21
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Blood samples were collected prior to, and 15, 30 and 60 days after intratesticular drug injection. Then all sera were removed by centrifuging the blood samples at 5000 rpm for 5 min, and stored at -20°C until assayed.

The serum testosterone level was determined by Coated-Tube Radioimmunoassay method using Active® Testosterone RIA DSL – 4000 kit (Diagnostic System Laboratories Inc. Texas, USA) in gamma counter (LKB-Wallac Multigamma) according to the report of the kit manufacturer. Briefly ; one vial of standard, labelled A, containing 0 ng/ml of testosterone was reconstituted with 1 ml deionised water. Other five vials of standards, labelled B-F, containing approximately 0.1, 0.5, 2.5, 10.0 and 25.0 ng/ml of testosterone were also reconstituted with 0.5 ml deionised water. Similarly two vials of controls, Levels I and II, containing low (0.3 ng/ml) and high (5.4 ng/ml) concentrations of testosterone were reconstituted with 0.5 ml deionised water, Then the sera were resolved in room temperature (~25°C), and liquid reagents and sera were mixed thoroughly by gentle inversion before use. Two plain uncoated tubes were labelled for Total Counts. All of the anti-testosterone-coated tubes were labelled and arranged in duplicate. Fifty μl of the standards, controls and sera was added to the bottom of appropriate tubes. Immediately 500 μl of testosterone [125I] reagent was added to the all tubes which were placed the test tube rack. The contents of tubes were mixed by shaking the test tube rack gently by hand. All tubes were incubated in a water bath at 37±2°C for 60 to 70 minutes. After the incubation, all tubes were decanted except Total Count Tubes by simultaneous inversion with a sponge rack into a radioactive waste receptacle. The tubes were stroked sharply on absorbent material to facilitate complete drainage and then allow them to drain on absorbent material for a minimum of 2 minutes. The tubes were then blotted to remove any droplets adhering to the rim before returning them to the upright position. As soon as the blotting, all of the tubes were counted in a gamma counter for one minute. The amount of testosterone was read in computer RIA data analysis program and expressed as ng/ml. The calibration range and sensitivity of kit were 0.1 to 25 and 0.08 ng/ml, respectively. The intra-assay and inter-assay coefficient variations of kit were lower than 9.6 % and 9.1 %, respectively.

HISTOPATHOLOGIC EXAMINATIONS :

The testicles were removed with open surgical technique 60 days after drug injection for histopathologic evaluation. The testis from each animal were fixed in formalin and embedded in paraffin wax. A section 5 μm thick was cut from the middle portion of each testis, stained with haematoxylin-eosin and examined under light microscopy at 200x magnification. The structures of the seminiferous tubules and interstitial spaces in the testis were examined.

STATISTICAL ANALYSES :

All data are presented as mean ± SEM (Standard Error of the Mean). The level of significance was set at p<0.05. Paired sample t-test was used to determine the differences between mean control and treatment values of mass activity, semen volume and sperm concentration. The

SEMEN COLLECTION AND EVALUATION :

Semen samples were collected using an artificial vagina prior to, and 15, 30 and 60 days after intratesticular drug injection. Semen volume was determined by direct reading the graduations of collection tubes [from 0.5 to 15 ml]. For determination of mass activity, non-cover slipped drop of fresh non-diluted semen was placed on a warm slide (37°C) under light microscope with heater stage at 100 x magnification. The condenser diaphragm of microscope was lowered in order to increase the contrast. The following descriptors were used for mass activity : [5] rapid dark swirls ; [4] slower dark swirls and eddies ; [3] little slower swirls ; [2] no swirls, but prominent individual cell motion ; [1] little individual cell motion ; and [0] no individual cell motion.

Sperm concentration was determined with a haemocytometer. Semen samples were decimally diluted with isotonic sodium citrate solution at 37°C [3 %, w/v dissolved in distilled water] at the rate of 1:10 for the determination of sperm motility. A slide was placed on light microscope with heater stage and allowed to warm up to 37°C, and then a small droplet of diluted semen was placed on the slide and the percentage of motile sperm was evaluated visually at a magnification of 400 x. Motility estimations were performed from 5 different fields in each sample. The mean of the five estimations was used as the final motility score. The abnormal sperm rate was determined from slides prepared with an Indian ink. A total of 300 sperm cells were counted on each slide under light microscope at 400 x magnification.

BLOOD COLLECTION AND TESTOSTERONE MEASUREMENT :

Blood samples were collected prior to, and 15, 30 and 60 days after intratesticular drug injection. Then all sera were removed by centrifuging the blood samples at 5000 rpm for 5 min, and stored at -20°C until assayed.

ULTRASOUND EXAMINATIONS :

Trans-scrotal testicular ultrasonography was performed with 7.5-5 MHz linear probe (Pie medical-scanvet 200) prior to the intratesticular injection and once a day for week following intratesticular injection and then one week intervals up to the end of the study. During this examination, the ultrasonographic appearance of a testis was evaluated and its height (h), width (W) and length (l) was measured. The volume of the testis was estimated using :

\[ \text{Volume} = (\text{I}_6/6) \times (w \times h \times l) \] equation.

Gauge needle directed from the caudal ventral aspect of each testis approximately 1 cm from the epididymal tail towards the dorsocranial aspect of the testis after restrained per animal. After 2 ml local anaesthetic (Citanest % 2) injections, 10 ml of absolute ethanol or 10 ml of a 30 % calcium chloride solution per testis was injected intra-testicularly.

HISTOPATHOLOGIC EXAMINATIONS :

The testicles were removed with open surgical technique 60 days after drug injection for histopathologic evaluation. The testis from each animal were fixed in formalin and embedded in paraffin wax. A section 5 μm thick was cut from the middle portion of each testis, stained with haematoxylin-eosin and examined under light microscopy at 200x magnification. The structures of the seminiferous tubules and interstitial spaces in the testis were examined.

STATISTICAL ANALYSES :

All data are presented as mean ± SEM (Standard Error of the Mean). The level of significance was set at p<0.05. Paired sample t-test was used to determine the differences between mean control and treatment values of mass activity, semen volume and sperm concentration. The
differences between control and treatment values for the percentages of motile and abnormal sperm were compared using a Chi-square ($\chi^2$) test. All the data were analyzed by using the SPSS (Version 12.0) and MNTAB® software package program (Version 14.0).

Results

Every animal tolerated the intratesticular injections of ethanol and calcium chloride. They did not suffer from any agitation and fever except for a slight increase of firmness of a testis on palpation. Food consumption remained unaffected among the two groups of animals.

Ultrasonographic examination following drug injection revealed that the testicular tissue of both group animals had a diffuse echotexture and increased echogenicity. The necrotized regions were differentiated ultrasonographically from the normal testicular tissue with a hypoechochogenic area (Figure 1). Testicular swelling was evident in both group bulls following injection and reached a peak within 48 hours. While testicular volume decreased significantly (P<0.05) in EG group after 3 weeks, no significant change occurred in CCG group (P>0.05). The testicles underwent atrophy by the 60th day in EG group while no marked alteration could be observed in CCG group (Figure 2). In two cases of the CCG group, testicular swelling was associated with orchitis and scrotal sloughing. At necropsy, their testicular tissues were found to have been necrotized. These problems were not observed in any animal of the EG group.

In this study, mean baseline value of serum testosterone concentration was 13.2 ± 2.6 ng/ml in EG group and 13.0 ± 2.4 in CCG group. After drug administration, these concentration values were significantly decreased in EG group (P<0.05) and remained almost constant in CCG group. Intratesticular administration of ethanol induced a gradual fall of serum testosterone concentrations from baseline 13.2 ± 2.6 ng/ml to 5.7 ± 3.9, 3.5 ± 0.7 and 2.6 ± 0.7 ng/ml on day 15, 30 and 60 after ethanol administration respectively.

The respective serum testosterone concentrations observed in the CCG group were 12.9± 1.8, 13.0 ± 2.2 and 12.9± 2.4 ng/ml on day 15, 30 and 60 after ethanol administration, respectively. Semen characteristics of only 3 bulls in ethanol group were very poor (p<0.01, p<0.001). Though only 3 bulls were infertile in ethanol injected group, other bulls were fertile. No statistically significant differences of semen characteristics were observed after calcium chloride treatment (Tables I and II).

Microscopic examination of testicles from ethanol treated bulls showed severe diffuse tubular necrosis along with varying degree of inflammatory response (Figure 3). Inflammatory cells consisted of mainly mononuclear cells. Interstitial oedema, fibrosis, haemorrhage were also detected. Some of the necrotic cells showed desquamation, or even calcification. Intertubular vessels were severely congested. Although similar lesions were noted in CCG treated bulls, the severity and distribution of the lesions were not so pronounced as in EG group.

Discussion

Chemical, surgical, and mechanical castration were previously used in bulls [16,28]. For chemical castration in male ruminants intratesticular lactic acid [16] tannic acid and zinc sulphate [10] alpha-hydroxypropionic acid [4], formalin [21] Castrate-Quin 14 [28] have been experimented. A few studies on the clinical use of absolute ethanol have been reported. These studies involved in the treatment of simple renal cysts, tumours and renal angioinfarction [7,20] and benign prostate hyperplasia [31]. The actual physiological effect of this treatment has previously been speculated. Nercosis and infarction leading to fibrosis, shrinkage, or sloughing of tissue are presumed to be due to its mechanism of action [2,31]. In our study ; severe diffuse tubular necrosis along with varying degree of inflammatory response were observed as a main finding (Figure 4).

B-mod ultrasonography was shown to produce adequate image quality to evaluate size and tissue changes. Measurements of testicular size have been used in the bull [23]. Similar measurements have been suggested as being of value in the dog [17]. Our study evaluated ultrasonographically the size and structural alterations in the testicle tissue following drug injection. Ultrasound imaging provides a rapid and accurate method of measuring testicular volume. Using this approach, we have detected a diffuse and increased echogenicity in the testicle tissue at acute period of drug administration, which was replaced by a decreased echogenicity in association with inflammatory reactions and necrotic formation.

For chemical sterilization ethanol and formaldehyde mixture were also used [12,24]. Gardner [1980] used intratesticularly a solution consisting of 3.6 % formaldehyde in 90 % ethanol for sterilization purpose in 10 bulls ; 3 among them showed active sperm after 85 days. We previously used a dose of 7 ml ethanol in male dogs [3] and judged the result as satisfactory. Therefore, we thought that the use of 10 ml drug in bulls because of their larger testicle sizes compared with dogs could be enough. In the present study, absolute ethanol was used in 6 bulls ; 3 bulls became infertile while the others maintained fertile despite marked testicular atrophy. This situation was attributed to the differences between the weight of testicle and testicle weights of the bulls and their specific susceptibilities.

Optimal effective doses of calcium chloride in rat were considered to range between 10-20 mg/kg, however, the best results were obtained using 20 mg/kg [18]. The former authors and others [18,26] found marked necrosis in the seminiferous tubules following the use of this agent. In a study, calcium chloride intratesticular administration was shown to reduce serum testosterone level in bulls [22]. The results of the present study, however disagree with the former one, since we could not evidenced marked alterations of serum testosterone levels and histopathological damages in testicles from CCG treated bulls. Thus, this agent appeared to be ineffective for chemo sterilization of bulls. On the other hand, in EG group, the serum testosterone levels declined markedly and meanwhile important findings were deter-
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References


TABLE I. Control and treatment values of mass activity, semen volume, sperm concentration, motility and abnormal sperm rate in bulls 60 days after intratesticular administration of ethanol. Data are presented as mean ± SEM.

<table>
<thead>
<tr>
<th>Bull Number</th>
<th>Mass activity</th>
<th>Semen volume (ml)</th>
<th>Sperm Concentration (….x 10^9 / ml)</th>
<th>Sperm Motility (%)</th>
<th>Abnormal Sperm Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treatment</td>
<td>Control</td>
<td>Treatment</td>
<td>Control</td>
</tr>
<tr>
<td>1008</td>
<td>4.0 ± 0.2</td>
<td>3.1 ± 0.3</td>
<td>4.8 ± 0.3</td>
<td>3.6 ± 0.9</td>
<td>0.90 ± 0.11</td>
</tr>
<tr>
<td>1010</td>
<td>3.6 ± 0.3</td>
<td>1.8 ± 0.8^a</td>
<td>4.5 ± 0.7</td>
<td>2.1 ± 0.3</td>
<td>1.05 ± 0.26</td>
</tr>
<tr>
<td>1012</td>
<td>4.1 ± 0.3</td>
<td>1.9 ± 0.7^a</td>
<td>4.6 ± 0.7</td>
<td>2.0 ± 0.4^a</td>
<td>0.91 ± 0.25</td>
</tr>
<tr>
<td>1014</td>
<td>4.2 ± 0.5</td>
<td>3.4 ± 0.2</td>
<td>4.3 ± 0.8</td>
<td>3.5 ± 0.8</td>
<td>0.98 ± 0.24</td>
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<tr>
<td>1016</td>
<td>3.9 ± 0.2</td>
<td>1.3 ± 0.6^a</td>
<td>4.5 ± 0.7</td>
<td>2.0 ± 0.2^a</td>
<td>0.90 ± 0.2</td>
</tr>
<tr>
<td>1018</td>
<td>4.3 ± 0.3</td>
<td>3.8 ± 0.3</td>
<td>4.5 ± 0.2</td>
<td>3.9 ± 0.2</td>
<td>0.86 ± 0.3</td>
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<tr>
<td>Mean</td>
<td>4.02 ± 0.1</td>
<td>2.55 ± 0.41</td>
<td>4.53 ± 0.06</td>
<td>2.85 ± 0.36</td>
<td>0.93 ± 0.02</td>
</tr>
</tbody>
</table>

*: p<0.01 versus control, **: p<0.001 versus control.

No significant differences were observed between control and treatment (p>0.05).

TABLE II. Control and treatment values of mass activity, semen volume, sperm concentration, motility and abnormal sperm rate in bulls 60 days after intratesticular administration of calcium chloride. Data are presented as mean ± SEM.

<table>
<thead>
<tr>
<th>Bull Number</th>
<th>Mass activity</th>
<th>Semen volume (ml)</th>
<th>Sperm Concentration (….x 10^9 / ml)</th>
<th>Sperm Motility (%)</th>
<th>Abnormal Sperm Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treatment</td>
<td>Control</td>
<td>Treatment</td>
<td>Control</td>
</tr>
<tr>
<td>1020</td>
<td>4.5 ± 0.2</td>
<td>4.3 ± 0.2</td>
<td>4.5 ± 0.2</td>
<td>4.2 ± 0.3</td>
<td>0.93 ± 0.02</td>
</tr>
<tr>
<td>1021</td>
<td>4.2 ± 0.3</td>
<td>4.3 ± 0.4</td>
<td>4.6 ± 0.5</td>
<td>5.0 ± 0.5</td>
<td>0.91 ± 0.25</td>
</tr>
<tr>
<td>1022</td>
<td>3.8 ± 0.5</td>
<td>4.0 ± 0.3</td>
<td>5.0 ± 0.9</td>
<td>5.2 ± 0.8</td>
<td>1.08 ± 0.35</td>
</tr>
<tr>
<td>1024</td>
<td>4.0 ± 0.7</td>
<td>3.9 ± 0.2</td>
<td>5.3 ± 0.4</td>
<td>4.8 ± 0.5</td>
<td>0.96 ± 0.22</td>
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<tr>
<td>1025</td>
<td>4.6 ± 0.1</td>
<td>3.8 ± 0.9</td>
<td>6.5 ± 0.6</td>
<td>6.0 ± 0.4</td>
<td>1.12 ± 0.21</td>
</tr>
<tr>
<td>1026</td>
<td>3.6 ± 0.4</td>
<td>3.5 ± 0.5</td>
<td>5.8 ± 0.2</td>
<td>5.2 ± 0.3</td>
<td>0.88 ± 0.28</td>
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<tr>
<td>Mean</td>
<td>4.12 ± 0.16</td>
<td>3.97 ± 0.12</td>
<td>5.28 ± 0.31</td>
<td>5.07 ± 0.24</td>
<td>0.98 ± 0.04</td>
</tr>
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</table>

In 60% of bulls, infertility rate was 50% ; we postulate that this rate could have been improved if this process had been carried on much younger bulls. The method of chemo sterilization based on ethanol intratesticular administration is readily applicable with no major harms and adverse effects. If 50% rate is considered satisfactory it can be used then in large flocks, in particular where no chance of operation exists.


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