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Oleuropein Effectively Improves the Quality of Pig Sperm at 17°C

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Abstract

Artificial Insemination (AI) has been widely used in pig reproduction because of its low price and high efficiency. Cryopreservation of sperm not only leads to a sharp decline in sperm motility, but had high price in pig reproduction. Therefore, most of farms stored pig sperm at 17°C. During the preservation of sperm, the accumulation of Reactive Oxygen (ROS) is the main reason for the decline of sperm motility. The aim of our study was to reduce the accumulation of ROS and improve sperm quality by adding oleuropein (OLE, CAS No. 32619-42-4) during the storage. The sperm samples were collected and diluted with different concentrations of oleuropein (0, 12, 24, 36, 48, 60 μmol/L). Sperm motility, plasma membrane integrity, acrosome integrity, sperm Total Antioxidant Capacity (T-AOC), Malondialdehyde (MDA) content, Catalase (CAT) activity and Reactive Oxygen Species (ROS) content of boar sperm were measured. The results suggested that adding 36μM of OLE samples significantly improved sperm quality during preservation at 17°C.

Keywords: Sperm preservation; Oleuropein; Artificial insemination; Pig

Abbreviations

OLE: Oleuropein; AI: Artificial Insemination

Introduction

Liquid sperm and Artificial Insemination (AI) are the most widely used technology in pig reproduction [1]. Pig sperm is extremely sensitive to temperature changes, and the cold stimulation of the sperm caused by low temperature will cause a sharp decline in sperm motility [2]. Moreover, due to the low economic effect of frozen sperm, its application in production is limited. In present, the preservation of pig sperm is mainly carried out at 17°C, because it is outstanding performance [3]. The main problem in preservation is the accumulation of Reactive Oxygen Species (ROS). While low concentrations of ROS play an important role in sperm capacitation, acrosome reaction, and mitochondrial respiration [4]. However, sperm itself has a limited antioxidant capacity [5]. After dilution, the antioxidant capacity in the sperm decreases, and the excess ROS produced by the sperm eventually exceeds its own antioxidant capacity [6].

A large number of studies have shown that antioxidants help to reduce cellular oxidative stress and reduce the production of oxidative free radicals [7]. During the sperm preservation process, the oxygen free radicals attack the plasma membrane of sperm, damage DNA, and mitochondrial membrane eventually lead to decreased sperm motility [8]. Besides, the extender supplementation with antioxidants could reduce the impact of oxidative stress caused by ROS during sperm preservation [9]. Antioxidants in extender play major roles in removing free radicals during sperm preservation [10]. Oleuropein is a phenolic substance with potent anti-oxidation activity, and has demonstrated favorable antibacterial and anti-oxidation performance in addition to having many other excellent physiological characteristics [11,12]. It has been reported that oleuropein not only prevents the production of DPPH (1,1-diphenyl-2-picrylhydrazyl, very stable nitrogen-centered free radical and commonly used for the evaluation of antioxidant activity *in vitro*) and hydrogen peroxide free radicals, but also could scavenge free radicals [13]. Olive leaf extracts also provide oxidative stress and enzymatic and non-enzymatic oxidation in diabetes in humans [14]. Other studies have shown that oleuropein can reduce DNA damage during sperm *in vitro* [15]. Therefore, we carried out this experiment to solve the oxidative stress damage of pig sperm in sperm preservation at 17°C.

The aim of our study was to evaluate the effects of different concentration of OLE to sperm diluent and assess the value of OLE in the preservation of sperm at 17°C. Our results showed that OLE could significantly improve the pig sperm quality at 17°C.

Materials and Methods

Ethics approval and consent to participate

The study was approved by the Institutional Animal Care and Use Committee of Northwest A&F University (Yangling Shaanxi, China). All operations were carried out according to the university's guidelines for animal research.

Animal and sperm collection

The experimental animals were ten adult male Yorkshire pigs (2-2.5 years old) with healthy records from the experimental farms of the Northwest A&F University. The boar sperm was collected using the hand-collecting method. Briefly, the collection cup was warmed to 35-37°C and was covered with a sterile filter paper. A sperm sample was collected from the ejaculated and the gelatinous fraction of the ejaculate was removed by filtration. Sperm samples were observed under a microscope and sperm motility greater than 75% were retained. Each of the control and treatment samples was repeated at least 5 times. The density was determined by the sperm

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density meter (Ibersan Co., Netherlands). The sperm was then diluted in diluents containing each concentration of oleuropein (12, 24, 36, 48, 60 μmol/L) to 1×10^8/ml.

Extender preparation

The basic diluent used throughout this study was composed of 37.5g fructose, 6.8g sodium citrate, 1.5g EDTA, 0.8g sodium bicarbonate, 0.3g citric acid, 0.7g KCl, and 1.0g beta cyclodextrin diluted in 1L deionized water. All reagents used in this study were sourced from Solarbio Co. (Beijing, China). Oleuropein (99.01% purity as determined by mass spectrometry, Desite Co., Chengdu, China) was accurately weighed and added to the above described media.

Osmotic pressure assay

The osmotic pressure was measured with the osmotic pressure detector. Regardless of calibration or measurement, the sample must be the same amount to get accurate results.

Sperm motility assay

The fresh sperm samples collected from boars, as well as the experimental diluents, were stored at room temperature for 0-5 days. A total of 3 different batches were tested, and the minimum number of spermatozoa analyzed was 1000 cells.

To accurately and reputably assess the motility of the sperm, a computer-assisted sperm quality analyzer (CASA) was used to calculate the sperm motility index in sperm. Details of the test statistic and test procedure can be found at HVIEW-SSAV8.0 (Hongshiye Co., Fujian, China). Using the automatic inspection system of boar sperm quality (CASA), the sperm samples with low sperm motility was eliminated, and the initial motility of the sperm in the sperm sample was above 75%. Sperm samples were then separated into different treatment groups. A 1ml aliquot of each sperm sample was transferred into a PCR tube, and incubated for 5min in a 37°C water bath. Next, 8μL of the test sperm was then placed on a pre-heated glass slide, a cover slip was added, and incubated at 37°C on a slide heating plate. The microscope fields were selected, and the sperm quality was detected and analyzed using the CASA system. The sampling parameters of CASA randomly analyzed for each sample 5 different fields, each containing a minimum of 100 sperm.

Analysis of plasma membrane and acrosome integrity

Acrosome integrity of porcine sperm was assessed, and the sperm acrosome integrity rate was calculated. This test was based on a FITC-PNA staining method and described by Aboagla and Terada [16]. After mixing the sperm, a 30μL aliquot was gently and evenly applied to the pre-treated slides, were air-dried, and fixed with anhydrous methanol for 10 min. After fixation, the sperm acrosomes were treated with the FITC-PNA working solution, and incubated at 37°C for 30min. After the incubation, the excess dye was washed from the slides 3 times for 10 minutes each with PBS. Coverslips were mounted, and fluorescence was stimulated with 450-490nm blue light on a fluorescence microscope (Lecia, Co., DMI8, Germany). Fluorescence was observed and photographed. A total of 5 fields were photographed for each sample, each field containing a minimum of 200 sperm.

Sperm plasma membrane integrity was assessed using the SYBR-14/Propidium Iodide (PI) dual labelling method. The samples (100μL) were placed into PCR tubes allowed to equilibrate to 37°C in a water bath for 5min. Next, 0.1μL of thawed SYBR-14 was added to each sample and the mixture was incubated in a 37°C water bath for 10min. Then, 0.5μL of PI was added to each sample, and incubated at 37°C for 10min. The sperm plasma membranes in the stained sperm were analysed using an upright fluorescence microscope (Lecia, Co., DMI8, Germany). A minimum of 1000 sperm were photographed, and the images were analyzed using the ImageJ software (National Institutes of Health, Bethesda, Maryland USA).

Analysis of antioxidant ability

The MDA was assessed using a malondialdehyde test kit (A003) (Nanjing Institute of Bioengineering, Co., Nanjing, China) according to the manufacturer's instructions. The tubes were then sealed with plastic wrap and punctured with a small needle to produce a small hole. The samples were then incubated in a 95°C water bath. After 40min incubation time, the water was cooled, centrifuged at 1500g for 10min. The supernatants were collected and absorbance was measured at 532nm in a fluorescent microplate reader (Boster, Co., USA).

The CAT activity was determined using a catalase test kit (A007) from the Nanjing Institute of Bioengineering. The sample preparation procedure for both CAT and T-AOC was conducted as outlined above for the MDA test.

ROS production

The amount of ROS in sperm cells was detected by DCFH-DA staining [17]. The experiment was performed in accordance with the instruction manual provided with the Active Oxygen Detection Kit (Nanjing Institute of Bioengineering, Co., Nanjing, China). Sperm suspensions (2×10^7 spermatozoa/mL) were incubated with 10μM DCFH-DA at 37°C for 30min in the dark. Sperm was rinsed three times with diluent, and the DCFH-DA dye solution that had not entered the sperm was thoroughly rinsed. Then, the sperm was re-suspended in the dilution. The fluorescence intensity was measured using a multifunctional fluorescence microplate reader at Ex/Em = 485/525nm. The sperm density in each treatment group was counted using a sperm density meter.

Statistical analysis

Data are presented as means ± SD. Differences between treatment groups were analyzed using a one-way ANOVA, followed by a Bonferroni post hoc test using the GraphPad software (Graph Pad, SanDiego, Co., CA, and USA). Differences were considered to be statistically significant at $p < 0.05$.

Results

Oleuropein treatment samples increased the motility of boar sperm

As shown in Table 1, oleuropein had little effect on the osmotic pressure of sperm storage ($p < 0.05$). A relatively balanced osmotic pressure was maintained throughout the culture period.

The results showed that the sperm motility of the 0 12, 24, 36, 48, 60 μmol/L oleuropein treatment groups were significantly higher than that of the control group ($p < 0.001$). On day 1, the motility of the control group was 69.85%, whereas that of the 36μmol/L oleuropein

Table 1: Osmotic pressure of each group during storage.

Oleuropein (μmol/L)	Conservation time (days)			
	0	1	3	5
NC	300.00 ± 1.00 ^{b}	301.00 ± 0.58 ^{b}	300.00 ± 0.00 ^{b}	301.05 ± 1.08 ^{ab}
12	300.37 ± 0.58 ^{ab}	301.00 ± 0.06 ^{b}	301.00 ± 1.00 ^{ab}	300.32 ± 0.56 ^{ab}
24	300.34 ± 0.59 ^{ab}	300.33 ± 0.58 ^{ab}	301.34 ± 0.58 ^{ab}	301.67 ± 1.15 ^{a}
36	302.00 ± 1.00 ^{ab}	301.65 ± 1.15 ^{a}	301.33 ± 0.58 ^{ab}	301.98± 1.04 ^{ab}
48	303.00 ± 1.07 ^{a}	301.43 ± 0.58 ^{a}	302.00 ± 1.00 ^{a}	300.33 ± 0.57 ^{ab}
60	302.01 ± 1.00 ^{ab}	301.74 ± 1.15 ^{a}	300.36 ± 0.58a ^{b}	300.00 ± 1.01 ^{b}

Note: Different superscripts within the same row demonstrate significant differences ($p < 0.05$). All treatments were replicated 10 times. Results are mean ± SD.

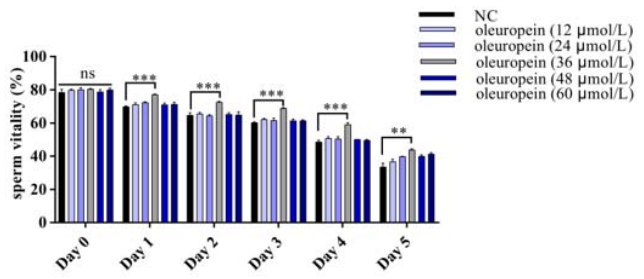

Figure 1: The effect of adding different concentrations of oleuropein on the first day to the 5th day on porcine sperm motility from CASA.
Note: Day 0 indicates two hours after oleuropein treatment. Ns signifies $p > 0.05$; *Significant at $p < 0.05$; **Significant at $p < 0.01$; ***Significant at $p < 0.001$. Results are mean ± SD.

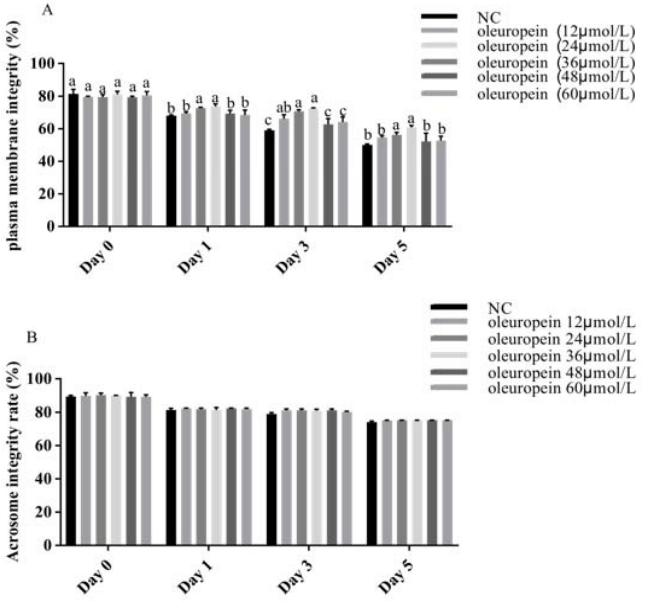

Figure 2: The effect of adding optimal concentration of oleuropein on the integrity rate of boar sperm plasma membrane and acrosome.
Note: Different superscripts within the same row demonstrate significant differences ($p < 0.05$). Results are mean ± SD.

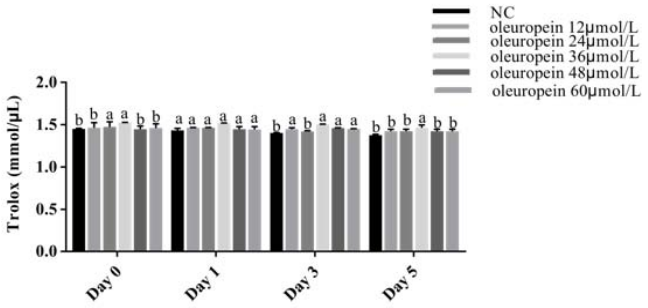

Figure 3: The effect of adding optimal concentration of oleuropein on the T-AOC of boar sperm.
Note: Different superscripts within the same row demonstrate significant differences ($p < 0.05$). Trolox is a standard for total antioxidant capacity. Results are mean ± SD.

treatment group was 77.25%. On day 4, the sperm motility of the 36μmol/L oleuropein treatment group was 59.11%, which was also significantly higher than that of the control group ($p < 0.001$). By day 5, the sperm motility of the 36μmol/L oleuropein treatment group was 43.86% and the sperm motility of the control group was 33.63%. It indicated that the addition of 36μmol/L oleuropein during the storage of boar sperm at room temperature could significantly improve sperm motility and prolong storage time (Figure 1).

Oleuropein treatment protected both the plasma membrane and acrosome integrity

Figure 2 showed us the rates of plasma membrane and acrosome integrity of boar sperm stored in oleuropein supplemented buffer. From the first day of preservation, 36μmol/L oleuropein treatment group and the other groups opened the gap, the integrity of the plasma membrane was significantly higher than the control group ($p < 0.05$), and the complete rate was also higher than the other groups. Furthermore, over the first 3 days of the test, the plasma membrane integrity rate of the treatment group was maintained above 70% (Figure 2A). However, with respect to acrosome integrity, although no significant differences were observed between the treatment and the other groups at any of the time points tested ($p > 0.05$), the acrosome integrity of the treatment group was always higher than the control group (Figure 2B).

Oleuropein treatment increases the T-AOC of sperm at 17°C storage

The effect of oleuropein treatment on boar sperm T-AOC activity was presented in Figure 3. After 1 day of storage, all groups exhibited reduced T-AOC activity. In addition, the T-AOC activity of the all treatment groups were significantly higher than that of the control group on days 3 of storage ($p < 0.05$). In the first 3 days of treatment, the total antioxidant capacity of the treatment group was significantly improved over that of the control group ($p < 0.05$). Furthermore, 36μmol/L oleuropein treatment group, the total antioxidant capacity

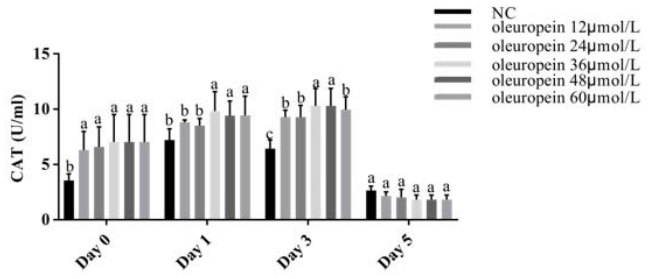

Figure 4: The effect of adding optimal concentration of oleuropein on CAT activity of boar sperm.
Note: Different superscripts within the same row demonstrate significant differences ($p < 0.05$). Results are mean ± SD.

of the treatment group was significant higher than that of the control group throughout the study period ($p < 0.05$).

Oleuropein treatment increased the CAT activity of boar sperm

In Figure 4, the effect of oleuropein treatment on CAT activity was presented. In the first 3 days of the experiment, the catalase activity of the treated group was significantly higher than that of the control group ($p < 0.05$). Although the sperm peroxidase enzyme treatment group and the control group was reversed on day 5, there was little difference between the groups; the sperm motility was low at this time.

Oleuropein treatment reduced the MDA content of boar sperm

Levels of MDA were measured as a marker of lipid peroxidation. In Figure 5, the effect of oleuropein treatment on sperm lipid peroxidation activity was presented. In the first day of treatment, the difference between the treatment and control groups was not significant ($p > 0.05$). While not statistically significant, the MDA concentration in the treatment group was observed to be lower than that of the control group ($p > 0.05$). From the day after treatment, the MDA content of the control group increased significantly, while the MDA content of each group in the treatment group was relatively low, and the 36μmol/L oleuropein treatment group remained at a low level ($p < 0.05$).

Discussion

During the normal and life sustaining metabolic processes within a cell or organism, a variety of free radicals are produced, including Reactive Oxygen Species (ROS). These radicals can cause damage to the body's cells. Among the radical species, ROS is the most common cause of sperm peroxidation damage [18]. Peroxidation of sperm may result in changes to the membrane structure of the cells, resulting in abnormal cellular functioning, as well as mitochondrial and DNA damage [19]. ROS usually makes sperm lose exercise capacity, affecting boar sperm quality and sperm preservation. These pathologic changes resulting from the accumulation of ROS can result in reduced motility, quality, and viability during preservation of the sperm. At present, it is believed that diluted antioxidants can protect the sperm plasma membrane from environmental influences. It is well established that antioxidants resist peroxidative damage caused by free radicals, especially ROS. Because of the potential

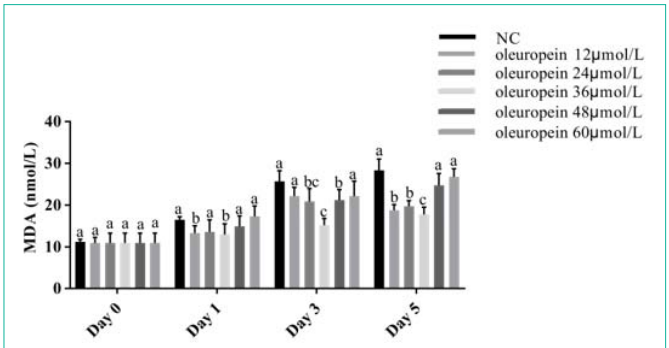

Figure 5: The effect of adding the optimal concentration of oleuropein on MDA of boar sperm.
Note: Different superscripts within the same row demonstrate significant differences ($p < 0.05$). Results are mean ± SD.

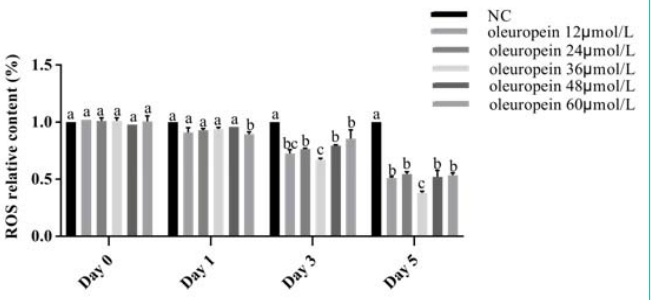

Figure 6: The effect of adding the optimal concentration of oleuropein on ROS of boar sperm.
Note: Different superscripts within the same row demonstrate significant differences ($p < 0.05$). Results are mean ± SD.

commercial benefit of this phenomenon to the swine industry, the use of antioxidants in sperm preservation media has been the focus of much research on boar sperm [20]. In such cases, antioxidants are added into extender to decrease the harmful effects of ROS on sperm quality [21]. The effects of extender containing different antioxidants on boar sperm and sperm quality are well documented [21,22].

Oleuropein is a diluent additive that exhibits a high level of antioxidant activity. However, the application of oleuropein in sperm preservation has not been reported at the time of publication. There are many issues limiting the successful preservation of mammalian sperm. In the present study, sperm motility of all groups were evaluated every day. The results suggested that sperm motility of the groups treated with oleuropein were higher than that of the control group. T-AOC activity, MDA content and H_2O_2 content of boar sperm were measured and analyzed during liquid preservation in the presence of different concentrations of oleuropein treatment. T-AOC activity is an indicator of the antioxidative capacity of sperm [21]. MDA is one of the by-products of lipid peroxidation; it has been used in various biochemical assays to monitor the degree of peroxidative damage sustained by spermatozoa [23]. H_2O_2 is a cause for the decline in sperm quality generated by sperm during the process of storage [24]. Therefore, the activity of sperm catalase was used in this study to judge its ability to decompose hydrogen peroxide. In our study, the endogenous antioxidant system of sperm under the protection of antioxidants has been greatly exerted. The decrease of MDA content and the improvement of plasma membrane integrity indicate that the

degree of membrane peroxidation is lower than that of the control group, ROS cumulative reduction, catalase activity was maintained at a higher level than the control group ($p < 0.05$). This result is similar to the results of the Büyükbalci's study, which is able to observe that this beneficial effect was the result of enhanced clearance of endogenous ROS, reduced lipid peroxidation, reduced concentrations of MDA, decreased serum lactate dehydrogenase, decreased creatine kinase activity, and increased myocardial activity [25].

We further examined the accumulation of sperm intracellular ROS as the trial progressed (Figure 6). We found intracellular ROS production was less in treated groups than in the control group ($p<0.05$), and mitochondrial activity was improved, which effectively improved the quality of sperm preservation. A decrease in ROS content means that the degree of attacking the membrane is reduced and the integrity of the sperm membrane system is protected. Plasma membrane integrity and acrosome integrity have fundamental roles in the fertilization process [26]. During the preservation process, free radicals and ROS in the sperm increase, causing oxidative stress, which leads to the peroxidation of unsaturated fatty acids in the sperm plasma membrane and acrosome membrane structure. In the present study, values for sperm quality variables, were determined including the sperm plasma membrane integrity and acrosome integrity, during boar sperm preservation at 17°C to verify the protective effects of oleuropein. Both the sperm plasma membrane integrity and acrosome integrity of the oleuropein group were greater than those of the control group (Figure 2) ($p < 0.05$). These data indicated that the addition of an appropriate amount of oleuropein has a beneficial effect on the sperm plasma membrane integrity and acrosome integrity.

After confirming, the fact that oleuropein has the effect of improving the preservation of pig sperm, we carried out a verification test of artificial insemination, and the treated sperm was subjected to an artificial insemination test together with the control group, and the researcher passed the test mother at 40 days. The pigs were subjected to ultrasound examination of pregnancy, and we obtained the results. From this result, we can see that the pregnancy rate of pig sperm treated with oleuropein was significantly higher than that of the control group after artificial insemination ($p < 0.05$). Based on the results of the previous experiments, we speculated that this might benefit from the maintenance of porcine spermicidal activity on pig sperm. The increase in sow pregnancy rate in this study is an important finding. For large-scale pig farms, this means less labor time and higher sow pregnancy rates, which also reduces production costs and saves production processes.

Conclusion

In summary, oleuropein maintains the motility of liquid-storage sperm, protects the plasma membrane and acrosome integrity of sperm, and enhances their antioxidant capacity. Oleuropein is able to reduce the ROS produced by pig sperm preservation, which has potential utilization value.

Declaration

Ethics statement: All of the animal experiments were conducted in accordance with the Chinese Guidelines on the Review of Welfare and Ethics of Laboratory Animals. All animal procedures were conducted with a protocol approved by the Animal Ethics Committee of the Northwest A&F University.

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