รายงานวิจัยฉบับสมบูรณ์

โครงการ: ผลของ Equex STM paste และการเจือจางน้ำเชื้อต่อการมีชีวิตของตัวอสุจิหลังการละลายน้ำเชื้อแช่แข็งในหลอดพลาสติคในแมวบ้าน *(Felis catus)*

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กันยายน 2549

สัญญาเลขที่ MRG4780201

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(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกอ. และ สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

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The effect of Equex STM paste and post-thaw dilution rates on sperm survival after thawing of frozen cat spermatozoa in straws

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Abstract

Spermatozoa are exposed to the potential of lysis due to ice crystal formation as well as to osmotic stress during freezing and thawing. The objective was to evaluate if post-thaw dilution or thwing temperature would affect quality and longevity of ejaculated cat spermatozoa. Six ejaculates were obtained from each of six male cats with 2 weeks interval. The semen from each ejaculate was frozen in four of half filled 0.25 mL straws. Straws were thawed 1) at 37 °C for 15 s and diluted with 0.25 mL Tris buffered, 2) at 37 °C for 15 s without dilution, 3) at 70 °C for 6 s and diluted with 0.25 mL Tris buffered, 4) at 70 °C for 6 s without dilution. Sperm motility, progressive motility, membrane integrity (SYBR-14/EthD-1 staining) and acrosome integrity (FITC-PNA/PI staining) were evaluated after collection and at times 0, 2, 4 and 6 hours post-thaw. The different treatments were analyse with GLM and pairwise comparisons were made with a paired t-test. At time 0, mean motility ranged between 59.2 ± 11.1 and 65.4 ± 11.2%, mean progressive motility between 3.6 ±0.5 and 4.1 ± 0.4, mean membrane integrity between 63.8 ± 8.8 and 65.1 ± 14.7%, and mean acrosome intact between 39.6 ± 22.1 and 47.7 ± 20.2% for the different treatments. Thawing at 70°C for 6 s resulted in the best motility compared to the others ($P < 0.05$). Regardless of the different thawing temperatures, post-thaw dilution significantly improved motility, progressive motility and acrosome integrity ($P < 0.05$). Membrane integrity did not differ significantly between treatments. Equex STM paste can be used successfully as a cryoprotectant for ejaculated cat spermatozoa without deleterious sperm longevity. With the freezing protocol used in this study, it is preferable to thaw frozen ejaculated cat spermatozoa at 70 °C for 6 s. A post-thaw dilution with Tris buffered solution is favourable to sperm motility and acrosome integrity.

Keywords: feline, freezing, semen

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ผลของอีเควิกซ์ เอสทีเอ็ม เพสท์ และการเจือจางน้ำเชื้อต่อการมีชีวิตของตัวอสุจิหลังการละลายน้ำเชื้อแช่แข็งในหลอดพลาสติคในแมวบ้าน

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บทคัดย่อ

ในขณะทำการแช่แข็งและละลายน้ำเชื้อ ตัวอสุจิมีความเสี่ยงต่อการถูกทำลายโดยการผลึกน้ำแข็งและจากการเปลี่ยนแปลงออสโมลาริตี้ การศึกษานี้มีวัตถุประสงค์เพื่อทดสอบผลของอุณหภูมิและการใช้สารเจือจางในการละลายน้ำเชื้อแช่แข็งต่อคุณภาพและการมีชีวิตของตัวอสุจิแมวที่ได้จากการหลั่งน้ำเชื้อ ทำการรีดเก็บน้ำเชื้อแมวจำนวน 6 ตัว ตัวละ 6 ครั้ง ห่างกัน 2 สัปดาห์ นำน้ำเชื้อผ่านกระบวนการแช่แข็งในหลอดพลาสติคขนาด 0.25 มิลลิลิตรจำนวน 4 หลอดต่อน้ำเชื้อที่ได้จากการหลั่งแต่ละครั้ง ละลายน้ำเชื้อแช่แข็งในอ่างอุ่น 1) ที่ 37 องศาเซลเซียส 15 วินาที และเจือจางด้วยน้ำยาทริสบัฟเฟอร์ 0.25 มิลลิลิตร 2) ที่ 37 องศาเซลเซียส 15 วินาที โดยไม่เจือจาง 3) ที่ 70 องศาเซลเซียส 6 วินาที และเจือจางด้วยน้ำยาทริสบัฟเฟอร์ 0.25 มิลลิลิตร 4) ที่ 70 องศาเซลเซียส 6 วินาที โดยไม่เจือจาง ตรวจการเคลื่อนไหวตัวอสุจิ การเคลื่อนที่ไปข้างหน้า ความสมบูรณ์ของผนังเซลล์ (โดยใช้สีย้อมไซเบอร์-14 และ เอทธิเดียม-1) และความสมบูรณ์ของอะโครโซม (โดยใช้สีย้อมฟิทซี-พีเอ็นเอ และโพพิเดียมไอโอไดด์) หลังการเก็บน้ำเชื้อ และ ที่ 0 2 4 และ 6 ชั่วโมง หลังละลายน้ำเชื้อแช่แข็ง วิเคราะห์ความแตกต่างของข้อมูลโดยใช้สถิติจีแอลเอ็ม และเปรียบเทียบข้อมูลโดยใช้การทดสอบแบบจับคู่ ที่เวลา 0 ชั่วโมง ตัวอสุจิหลังละลายด้วยวิธีต่าง ๆ มีความเคลื่อนไหวเฉลี่ยร้อยละระหว่าง 59.2 ± 11.1 และ 65.4 ± 11.2 การเคลื่อนไหวไปข้างหน้าเฉลี่ยระหว่าง 3.6 ±0.5 และ 4.1 ± 0.4 ความสมบูรณ์ของผนังเซลล์เฉลี่ยร้อยละระหว่าง 63.8 ± 8.8 และ 65.1 ± 14.7 และความสมบูรณ์ของอะโครโซมเฉลี่ยร้อยละระหว่าง 39.6 ± 22.1 และ 47.7 ± 20.2 การละลายที่อุณหภูมิ 70 องศาเซลเซียส เป็นเวลา 6 วินาที ให้ผลร้อยละของการเคลื่อนไหวตัวอสุจิมากที่สุด ($P < 0.05$) การละลายน้ำเชื้อแช่แข็งโดยใช้สารเจือจางให้ผลการเคลื่อนไหวตัวอสุจิ การเคลื่อนที่ไปข้างหน้า และความสมบูรณ์ของอะโครโซมดีขึ้นในทุกอุณหภูมิที่ใช้ละลาย ($P < 0.05$) แต่ไม่มีความแตกต่างของความสมบูรณ์ของผนังเซลล์ อีเควิกซ์ เอสทีเอ็ม เพสท์ สามารถใช้เป็นสารป้องกันตัวอสุจิแมวที่ได้จากการหลั่งน้ำเชื้อในกระบวนแช่แข็งได้โดยไม่มีผลลบต่อการมีชีวิตของตัวอสุจิ กล่าวโดยสรุป หากจะใช้วิธีการแช่แข็งน้ำเชื้อในการศึกษานี้ ควรใช้การละลายที่อุณภูมิ 70 องศาเซลเซียส เป็นเวลา 6 วินาที โดยทำการเจือจางน้ำเชื้อขณะละลายด้วยสารละลายทริสบัฟเฟอร์ จะช่วยให้การเคลื่อนไหวของตัวอสุจิดีขึ้นและอะโครโซมถูกทำลายน้อยลง

คำสำคัญ: แมว การแช่แข็ง น้ำเชื้อ

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5. ระยะเวลาดำเนินงาน เดือนกรกฎาคม 2547 ถึง เดือนมิถุนายน 2549

(ได้รับอนุมัติให้ขยายเวลา3 เดือน เนื่องจากลาคลอดบุตร เป็นเดือนกันยายน 2549 ตามหนังสือที่ นร 6808/175/2549 ลงวันที่ 4 เมษายน 2549)

6. ได้เสนอโครงการนี้ หรือโครงการที่มีส่วนเหมือนกับเรื่องนี้บางส่วน เพื่อขอทุนต่อแหล่งทุนอื่นที่ใดบ้าง

☑ ไม่ได้เสนอต่อแหล่งทุนอื่น

☐ เสนอต่อ.....

ชื่อโครงการที่เสนอ.....

กำหนดทราบผล (หรือสถานสภาพที่ทราบ).....

7. ปัญหาที่ทำการวิจัย และความสำคัญของปัญหา

แมวบ้านเป็นต้นแบบในการศึกษาด้านระบบสืบพันธุ์ของสัตว์ป่าตระกูลแมวอื่น ๆ เพราะมีสรีรวิทยาของระบบสืบพันธุ์ที่พัฒนามาใกล้เคียงกัน (Wildt et al., 1986) โดยจะเห็นได้จากความสำเร็จของการผสมเทียมเสือชีต้าร์ *(Acinonyx jubatus)* (Howard et al., 1992) เสือไซบีเรียน *(Panthera tigris altaicia)* (Donoghue et al., 1993) เสือพูม่า *(Felis concolor)* (Barone et al., 1994) และเสือลายเมฆ *(Neofelis nebulosa)* (Howard et al., 1996) โดยใช้เทคนิคที่พัฒนามาจากการผสมเทียมในแมวบ้าน (Howard et al., 1992) ในบรรดาสัตว์ตระกูลแมว (Felidae) 38 ชนิด เป็นสัตว์ป่าหายาก (endangered species) ที่พบในประเทศไทย 9 ชนิด คือ เสือโคร่ง *(Panthera tigris)* เสือดาว *(Panthera pardus)* เสือลายเมฆ *(Neofelis nebulosa)* เสือไฟ *(Felis temmincki)* เสือปลา *(Felis viverrina)* แมวดาว *(Felis bengalensis)* แมวป่าหรือเสือกระต่าย *(Felis chaus)* แมวป่าหัวแบน *(Felis planiceps)* และแมวลายหินอ่อน *(Felis marmorata)* โดยในธรรมชาติไม่มีผู้พบเห็นแมวป่า แมวป่าหัวแบน และแมวลายหินอ่อนมาเป็นเวลานานแล้ว (ศลิษาและอลัน 2538) จึงได้รับการประกาศให้เป็นสัตว์ป่าสงวน ตามพระราชบัญญัติสงวนและคุ้มครองสัตว์ป่า ฉบับปี พ.ศ. 2535 สำหรับสัตว์ป่าตระกูลแมวส่วนใหญ่ ห้ามทำการซื้อขายหรือนำเข้าออกนอกราชอาณาจักรตามสนธิสัญญาว่าด้วยการค้าสัตว์ป่านานาชาติ (The Convention on International Trade in Endangered Species, CITES)

(CITES, 2000) จึงเป็นสาเหตุสำคัญที่ทำให้สัตว์ป่าซึ่งมีจำนวนน้อยมีการผสมพันธุ์เลือดชิดทั้งในกรงเลี้ยงและในป่า มีผลทำให้เกิดการเปลี่ยนแปลงทางพันธุกรรมต่าง ๆ ที่สำคัญได้แก่ ลักษณะผิดปกติของตัวอสุจิจำนวนมากกว่า 60 เปอร์เซ็นต์ในการหลั่งน้ำเชื้อหนึ่งครั้ง ซึ่งพบในกลุ่มประชากรแมวบ้านและสัตว์ป่าตระกูลแมวอื่น ๆ บางชนิด (teratospermic cats) ที่อยู่อาศัยในพื้นที่จำกัด (Howard et al., 1996; Pukazhenthi et al., 2001) ตัวอสุจิจากสัตว์เหล่านี้จะมีความทนทานต่อความเย็นและออสโมลาลิตี้ต่ำกว่าตัวอสุจิของสัตว์ที่มีการหลั่งจากน้ำเชื้อปกติ (normospermic cats) ซึ่งมีผลต่อการเก็บรักษาและการผสมติด (Pukazhenthi et al., 1999; Pukazhenthi et al., 2000)

นอกจากแมวบ้านจะมีความสำคัญในแง่ของการเป็นตัวแทนในการศึกษาโรคและความผิดปกติในคน และการศึกษาสรีรวิทยาการสืบพันธุ์สัตว์ป่าตระกูลแมวแล้ว แมวบ้านยังเป็นสัตว์เลี้ยงที่ได้รับความนิยมสูงรองจากสุนัข เพราะเลี้ยงได้ในที่อยู่อาศัยที่มีพื้นที่จำกัด ดังจะเห็นได้จากความนิยมแมวสายพันธุ์แท้ โดยเฉพาะแมวไทย 4 ชนิด ได้แก่ แมวสีสวาด แมววิเชียรมาศ แมวขาวมณี และแมวศุภลักษณ์ ซึ่งมีชื่อเสียงในเรื่องความโดดเด่นของสายพันธุ์ไปทั่วโลก อย่างไรก็ตาม ประชากรแมวที่ใช้ในการทดลอง แมวป่า และแมวพันธุ์แท้เหล่านี้มีจำนวนจำกัด การใช้เทคโนโลยีทางวิทยาการสืบพันธุ์โดยการเก็บรักษาน้ำเชื้อโดยการแช่แข็ง จึงมีประโยชน์ในด้านการเพิ่มจำนวนสัตว์ รวมถึงการกระจายพันธุกรรมเพื่อเพิ่มความหลากหลายของยีน (genetic diversity) ในหมู่สัตว์ที่อาศัยในพื้นที่เดียวกัน อันเป็นเป้าหมายสำคัญในการอนุรักษ์สัตว์ป่าใกล้สูญพันธุ์ แมวเลี้ยงพันธุ์แท้ และแมวที่มีพันธุกรรมที่ใช้เป็นต้นแบบในการทดลองความผิดปกติในคน

การศึกษาวิธีการแช่แข็งน้ำเชื้อรวมทั้งการละลายน้ำเชื้อแช่แข็งในสัตว์เหล่านี้ เพื่อให้ตัวอสุจิหลังการละลายมีชีวิตอยู่นาน เป็นการเพิ่มโอกาสการผสมติด เพราะกระบวนการแช่แข็งทำให้เกิดการลดลงของความเคลื่อนไหว การทำลายของผนังหุ้มเซลและอะโครโซมของตัวอสุจิอย่างมาก จึงลดระยะเวลาการมีชีวิตอยู่ของตัวอสุจิที่พร้อมปฏิสนธิ (Wood et al., 1993; Hay and Goodrowe, 1993; Pukazhenthi et al., 1999) ในขณะที่ช่วงเวลาการผสมเทียมด้วยน้ำเชื้อแช่แข็งที่เหมาะสมกับการตกไข่หลังการกระตุ้นโดยฮอร์โมนเอชซีจี (human chorionic gonadotrophin, hCG) ในแมวยังไม่มีการรายงานที่แน่ชัด และยังแนะนำให้ทำการผสมเทียมถึงสองครั้ง คือในวันที่ฉีดฮอร์โมนเอชซีจี และอีกสองวันถัดมา (Axnér and Linde-Forsberg, 1998) ซึ่งเป็นการสิ้นเปลืองน้ำเชื้อ และถ้าเป็นในสัตว์ป่าจะต้องทำการวางยาสลบถึงสองครั้งซึ่งเป็นการเสี่ยง ดังนั้นการมีชีวิตอยู่นานของตัวอสุจิหลังการละลายจึงมีความสำคัญอย่างยิ่ง

8. วัตถุประสงค์

1. เพื่อศึกษาผลของ Equex STM paste ซึ่งเป็นสารดีเทอร์เจ้น (detergent) ในการป้องกันการทำลายตัวอสุจิแมวจากกระบวนการแช่แข็ง
2. เพื่อหาระยะเวลาการมีชีวิตและคุณภาพของตัวอสุจิหลังการละลายน้ำเชื้อแช่แข็งโดยการใช้และไม่ใช้น้ำยาเจือจางที่อุณหภูมิ 37 และ 70 องศาเซลเซียส

9. ระเบียบวิธีวิจัย

สัตว์ทดลอง

แมวบ้าน *(Felis catus)* เพศผู้โตเต็มวัย จำนวน 6 ตัว มีสุขภาพแข็งแรงสมบูรณ์ มีคุณภาพน้ำเชื้อเป็นปกติ โดยดูจากการเคลื่อนไหวตัวอสุจิมากกว่า 60 เปอร์เซ็นต์ มีจำนวนตัวอสุจิลักษณะปกติมากกว่า 60 เปอร์เซ็นต์ต่อการหลั่งน้ำเชื้อหนึ่งครั้ง เลี้ยงแมวในกรงเลี้ยงเดี่ยวมีพื้นที่ไม่น้อยกว่า 1 x 1.5 เมตรต่อตัว ได้รับอาหารเม็ดสำเร็จรูปวันละ 1 ครั้ง และมีน้ำให้กินตลอดเวลา การเลี้ยงแมวและการทดลองผ่านการอนุญาตจากคณะกรรมการพิจารณาการใช้สัตว์ทดลอง ฝ่ายวิจัยและบริการทางวิชาการ คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

วิธีทดลอง

การรีดเก็บน้ำเชื้อแมวจำนวน 6 ตัว ตัวละ 6 ครั้ง ห่างกัน 2 สัปดาห์ โดยใช้เครื่องกระตุ้นไฟฟ้า นำน้ำเชื้อผ่านกระบวนการแช่แข็งในหลอดพลาสติคขนาด 0.25 มิลลิลิตรจำนวน 4 หลอดต่อน้ำเชื้อที่ได้จากการหลั่งแต่ละครั้ง ละลายน้ำเชื้อแช่แข็งในอ่างอุ่น 1) ที่ 37 องศาเซลเซียส 15 วินาที และเจือจางด้วยน้ำยาทริสบัฟเฟอร์ 0.25 มิลลิลิตร 2) ที่ 37 องศาเซลเซียส 15 วินาที โดยไม่เจือจาง 3) ที่ 70 องศาเซลเซียส 6 วินาที และเจือจางด้วยน้ำยาทริสบัฟเฟอร์ 0.25 มิลลิลิตร 4) ที่ 70 องศาเซลเซียส 6 วินาที โดยไม่เจือจาง ตรวจการเคลื่อนไหวตัวอสุจิ การเคลื่อนที่ไปข้างหน้า ความสมบูรณ์ของผนังเซลล์ (โดยใช้สีย้อมไซเบอร์-14 และ เอทธิเดียม-1) และความสมบูรณ์ของอะโครโซม (โดยใช้สีย้อมฟิทซี-พีเอ็นเอ และโพพิเดียมไอโอไดด์) หลังการเก็บน้ำเชื้อ และ ที่ 0 2 4 และ 6 ชั่วโมง หลังละลายน้ำเชื้อแช่แข็ง วิเคราะห์ความแตกต่างของข้อมูลโดยใช้สถิติจีแอลเอ็ม และเปรียบเทียบข้อมูลโดยใช้การทดสอบแบบจับคู่

10. ผลการทดลอง

ผลการทดลองพบว่าที่เวลา 0 ชั่วโมง ตัวอสุจิหลังละลายด้วยวิธีต่าง ๆ มีความเคลื่อนไหวเฉลี่ยร้อยละระหว่าง 59.2 ± 11.1 และ 65.4 ± 11.2 การเคลื่อนไหวไปข้างหน้าเฉลี่ยระหว่าง 3.6 ±0.5 และ 4.1 ± 0.4 ความสมบูรณ์ของผนังเซลล์เฉลี่ยร้อยละระหว่าง 63.8 ± 8.8 และ 65.1 ± 14.7 และความสมบูรณ์ของอะโครโซมเฉลี่ยร้อยละระหว่าง 39.6 ± 22.1 และ 47.7 ± 20.2 การละลายที่อุณหภูมิ 70 องศาเซลเซียส เป็นเวลา 6 วินาที ให้ผลร้อยละของการเคลื่อนไหวตัวอสุจิมากที่สุด ($P < 0.05$) การละลายน้ำเชื้อแช่แข็งโดยใช้สารเจือจางให้ผลการเคลื่อนไหวตัวอสุจิ การเคลื่อนที่ไปข้างหน้า และความสมบูรณ์ของอะโครโซมดีขึ้นในทุกอุณหภูมิที่ใช้ละลาย ($P < 0.05$) แต่ไม่มีความแตกต่างของความสมบูรณ์ของผนังเซลล์ อีเควิกซ์ เอสทีเอ็ม เพสท์ สามารถใช้เป็นสารป้องกันตัวอสุจิแมวที่ได้จากการหลั่งน้ำเชื้อในกระบวนแช่แข็งได้ โดยไม่มีผลลบต่อการมีชีวิตของตัวอสุจิ

11. สรุป

หากจะใช้วิธีการแช่แข็งน้ำเชื้อในการศึกษานี้ ควรใช้การละลายที่อุณภูมิ 70 องศาเซลเซียส เป็นเวลา 6 วินาที โดยทำการเจือจางน้ำเชื้อขณะละลายด้วยสารละลายทริสบัฟเฟอร์ จะช่วยให้การเคลื่อนไหวของตัวอสุจิดีขึ้นและอะโครโซมถูกทำลายน้อยลง

The effect of Equex STM paste and post-thaw dilution rates on sperm survival after thawing of frozen cat spermatozoa in straws

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Abstract

Spermatozoa are exposed to the potential of lysis due to ice crystal formation as well as to osmotic stress during freezing and thawing. The objective was to evaluate if post-thaw dilution or thawing temperature would affect quality and longevity of ejaculated cat spermatozoa. Six ejaculates were obtained from each of six male cats with 2 weeks interval. The semen from each ejaculate was frozen in four of half filled 0.25 mL straws. Straws were thawed 1) at 37 °C for 15 s and diluted with 0.25 mL Tris buffered, 2) at 37 °C for 15 s without dilution, 3) at 70 °C for 6 s and diluted with 0.25 mL Tris buffered, 4) at 70 °C for 6 s without dilution. Sperm motility, progressive motility, membrane integrity (SYBR-14/EthD-1 staining) and acrosome integrity (FITC-PNA/PI staining) were evaluated after collection and at times 0, 2, 4 and 6 hours post-thaw. The different treatments were analyse with GLM and pairwise comparisons were made with a paired t-test. At time 0, mean motility ranged between 59.2 ± 11.1 and 65.4 ± 11.2%, mean progressive motility between 3.6 ±0.5 and 4.1 ± 0.4, mean membrane integrity between 63.8 ± 8.8 and 65.1 ± 14.7%, and mean acrosome intact between 39.6 ± 22.1 and 47.7 ± 20.2% for the different treatments. Thawing at 70°C for 6 s resulted in the best motility compared to the others ($P < 0.05$). Regardless of the different thawing temperatures, post-thaw dilution significantly improved motility, progressive motility and acrosome integrity ($P < 0.05$). Membrane integrity did not differ significantly between treatments. Equex STM paste can be used successfully as a cryoprotectant for ejaculated cat spermatozoa without deleterious sperm longevity. With the freezing protocol used in this study, it is preferable to thaw frozen ejaculated cat spermatozoa at 70 °C for 6 s. A post-thaw dilution with Tris buffered solution is favourable to sperm motility and acrosome integrity.

Keywords: feline, freezing, semen

Introduction

Except for the domestic cat (*Felis catus*) and a few other felid species, the Felidae are listed as threatened by the Convention on International Trade in Endangered Species (CITES, 2000). Among nine species of felid in Thailand, one (*Felis marmorata*) is considered nearly extinct. The other eight species (*Panthera tigris, Panthera pardus, Neofelis nebulosa, Felis temmincki, Felis viverrina, Felis bengalensis, Felis chaus* and *Felis planiceps*) are deemed highly in risk of extinction. Cryopreservation of spermatozoa allows the preservation of genetic material from endangered wild species. Because wild felids are in risk of extinction, the domestic cat has been used as a model of these wild species for reproductive studies (Wild et al., 1986).

Cryopreservation induces a series of osmotic, chemical, and mechanical stresses to sperm, causing death of some spermatozoa, membrane and acrosome disruptions, and reducing fertility ability as a consequence (Watson, 1995). Several cryoprotocols have been experimented with the ultimate goal to minimize damages in cat spermatozoa (Platz et al., 1978; Wood et al., 1993; Lengwinat and Blottner, 1994; Zambelli et al., 2002; Axnér et al., 2004). During freezing and thawing, the spermatozoon is exposed to non-physiological concentrations of extracellular solutes. Addition and removal of cryoprotectant has been reported as the major factor altering cellular volume excursions during freezing process and subsequently causing sperm damages (Gao et al., 1997). During

addition, the spermatozoon transiently shrinks (loss of intracellular water across an osmotic gradient) and then returns to its near original volume upon cryoprotectant intracellular permeation (Gao et al., 1997). Significant volume change also occurs at thawing when the cell exchanges intracellular cryoprotectant for re-entry of water (Gao et al., 1995, Gao et al., 1997). Furthermore, the spermatozoon is exposed to the potential of lysis due to ice crystal formation during freezing and thawing. All these fators can stress and perturb the morphological architecture of the spermatozoon, disrupting membranes, the motility apparatus and survival itself. However, a proportion of rapidly cooled cells can be rescued by rewarming rapidly and slowly cooled cells can be rescued by rewarming slowly thus limiting the time for recrystallization to oocur (Mazur, 1984). Slow dilution after thawing prevents the sharp change in osmolarity and viscosity between the cryopreservation solution and diluent (Nakagata and Tadeshima, 1992).

Addition of Equex STM paste to a Tris extender has been reported to improve post-thaw viability, motility, acrosome integrity and longevity of frozen-thawed dog spermatozoa (Rota et al., 1997; Peña and Linde-Forsberg, 2000). In cat, Equex has a positive effect on the acrosomes but has a negative effect on the longevity of the epididymal spermatozoa after thawing (Axnér et al., 2004). However, ejaculated and epididymal spermatozoa have different membrane characteristics which may result in different susceptibility for cold shock (White, 1993). This study aimed to find the optimal temperature and condition for thawing frozen ejaculated cat spermatozoa.

Materials and methods

Animals

Six male cats aged 1-2 years, weighed 4-6 kg, were kept in individual stainless steel cages measuring 1.5x1.5x1.8 m (WxLxH) located in a confined room. The cats were provided the ambient light from the windows, a commercial feline diet (Science diet, Hill's Pet Nutrition Inc, USA) and free access to water.

Semen collection

Generalized anesthesia was induced using single bolus of 10 mg/kg of propofol (Fresofol 1%; Fresenius Kabi Austria GmbH, Graz, Austria) intravenously (Chatdarong et al., 2006). Electroejaculation was performed in the cats using a previously described protocol (Howard et al., 1986), consisting of a total of 80 stimuli (2-5 V) divided into three series, delivered through a rectal probe (1.3 cm diameter, 24 cm length) and an electrostimulator (AC, 60-Hz sine wave; PT Electronics, Boring, OR, USA). Ejaculate was immediately diluted 1:1 in Tris buffered solution. Aliquots of the sample were evaluated for sperm quality. Six ejaculates were obtained from each six male cats at 2 weeks interval.

Evaluation of sperm concentration and morphology

To determine sperm concentration, a 5 µL aliquot of the sample at room temperature was diluted in 200 µL of formol-saline and spermatozoa were counted in 25 squares of a haemocytometer chamber. For evaluation of sperm morphology, the combined use of wet smears and stained smears was used since abnormal sperm heads require different evaluation techniques than do other sperm abnormalities to get the most accurate counts of all sperm defects (Sekoni et al., 1981). Two hundred spermatozoa in the formol-saline were evaluated under a phase-contrast microscope at 1000x magnification. Morphology of the sperm heads was evaluated separately by counting 500 spermatozoa on carbol-fuchsine stained smears under a light microscope at 1000x magnification.

Evaluation of motility, membrane integrity and acrosome integrity

To evaluate sperm motility and progressive motility (scale of 0-5; Platz and Seager, 1978), an aliquot of the sperm sample was placed on a warm slide at 38 °C and a minimum of four fields were evaluated subjectively under a phase-contrast microscope at 200x. Plasma membrane integrity was evaluated with a combination of the fluorophores EthD-1 and SYBR-14 (Molecular probes Inc., OR, USA). Aliquots of 5 µL sperm suspension were mixed with 1 µL 0.38 µM SYBR-14 in DMSO (final concentration 0.054 µM) and 1 µL 14 µM EthD-1 in PBS (final concentration 2 µM) and

incubated in the dark at 37 °C for 30 min. Samples were evaluated with an epifluoresent microscope. A total of 200 spermatozoa were evaluated from each sample. Live spermatozoa stained green with SYBR-14 while dead spermatozoa stained red with EthD-1. Some moribound spermatozoa stained with both red and green were not reported. A fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA, Sigma, St.Louis, USA) stain was used. A 5 µL sperm suspension was smeared onto a micro-slide, and then sperm membranes were permeabilized with 95% ethanol for 30 s. After mixing 90 µL FITC-PNA (100 µg/mL in PBS) with 5 µL propidium iodide (PI, 340 µM in PBS, final concentration 18 µM), 20 µL of this mixture was spread over each smear. The slides were incubated in a moist chamber at 4 °C for 30 min then rinsed with 4 °C distilled water and air dried at 4 °C. The slides were evaluated using epifluoresent microscopy under a glycerine solution. Propidium iodide was used as a counterstain to give a better visualization of the spermatozoa. Two hundred spermatozoa were assessed in each smear and classified into three categories based on their FITC-PNA patterns (Cheng et al., 1996). Briefly, the acrosome region of sperm head displaying bright fluorescence was defined as intact acrosome. The acrosome region of sperm head displaying disrupted, patchy image of fluorescence was defined as reacted acrosome. The sperm head displaying equatorial segment was defined as acrosome loss.

Media used for freezing and thawing

The sperm-freezing media consisted of two formulas of extender. Extender 1 contained 2.4% (w/v) Tris (Fluka, Buenos Airez, Argentina), 1.4% (w/v) citric acid (Fluka, Buenos Airez, Argentina), 0.8% (w/v) glucose (BDH, VWR International, Poole, England), 3% (v/v) glycerol (Fluka, Buenos Airez, Argentina), 20% (v/v) egg yolk, 0.06% (w/v) Na-benzylpenicillin (M&H Manufacturing, Samudprakan, Thailand), 0.1% (w/v) streptomycin sulphate (M&H Manufacturing, Samudprakan, Thailand) in distilled water (pH 6.5, 833 mOsm). Extender 2 had the same omposition as that of Extender 1 except that it contained 7% glycerol (v/v) and 1% (v/v) Equex STM paste (Nova Chemical Sales, Scituate, Inc., MA, USA) (pH 6.5, 1630 mOsm). The thawing medium (Tris buffered) had the same composition as Extender 1 except that it did not contain egg yolk or glycerol (pH 6.5, 270 mOsm). Each freezing extender and thawing medium were prepared in one batch and stored frozen at -20 °C.

Freezing of spermatozoa

The semen sample was centrifuged at 300 x *g* for 6 min and the supernatant was discarded. The sperm pellet was extended with 250 µL of Extender 1. The extended sample was cooled in a bench cooler from a room temperature to reach 4 °C in about 45 min. The cooled sample was then diluted 1:1 with Extender 2. A 0.25 mL mini-straw was half filled with the extended semen. Before loading the extended semen, 10 µL of a 1:1 mixture of Extenders 1 and 2 was drawn into the straw to fill the cotton plug in the top of the straw according to Axnér et al (2004). Each sample was divided and fill into four straws. The straws were frozen as described by Rota et al. (1997). Briefly the straws were put into goblets and frozen by lowering the goblets into an Apollo SX-18 LN_2 tank (MVE Cryogenetics, New Prague, MN, USA) 16-18 cm filled with liquid nitrogen in three steps, at 7, 13 and 20 cm from the top of the tank, for 2, 2 and 1 min, respectively.

Thawing of spermatozoa

Four straws from each ejaculate were thawed in a water bath 1) at 37°C for 15 s and emptied into a prewarmed tube containing 250 µL of thawing medium 2) at 37°C for 15 s and emptied into a prewarmed tube 3) at 70°C 6 s and emptied into a prewarmed tube 4) without dilution at 70°C 6 s and emptied into a prewarmed tube containing 250 µL of thawing medium. Thereafter they were held in the dark at 38 °C. After 5 min at 38 °C (time 0) and 2, 4 and 6 h after thawing, aliquots were removed for evaluation of sperm motility, progressive motility, membrane integrity and acrosome status.

Statistical analyses

Means are expressed as means ± SEM. Data on post-thaw motility, plasma membrane integrity and acrosome integrity were analyzed using ANOVA (PROC GLM; SAS Institute Inc., Cary, NC, USA). A pair-wise t-test was applied to compare differences between the means of sperm

characteristics after thawing at different temperatures and with or without dilution. A value of $P < 0.05$ was considered statistically significant.

Results

Volume of ejaculates from all males averaged 64.5 ± 34.9 μL with sperm motility of 85.5 ± 6.8%, progressive motility of 4.7 ± 0.4, percentage of intact membrane of 88 ± 7.2 and percentage of intact acrosome of 74 ± 11.2. Total number of spermatozoa per ejaculate averaged 30.6 ± 23.6 x 10^6. The mean percentage of morphologically normal sperm head was 72.9 ± 8.7 and the mean percentage of morphologically normal midpiece and tail was 75.5 ± 7.5. Mean percentage of various morphological abnormalities of head, midpiece and tail was demonstrated in Table 1. Sperm characteristics were different between cats, between ejaculates and between times of incubation after thawing ($P < 0.05$). Freezing and thawing resulted in a significant reduction of spermatozoa with motility, progressive motility, intact membranes and intact acrosomes ($P < 0.05$) (Table 2, 3, 4, and 5). Thawing at 70°C 6 s and diluted with Tris buffered resulted in the best motility compared to the others ($P < 0.05$).The post-thaw dilution significantly improved the sperm motility, progressive motility and percentages of intact acrosomes ($P < 0.05$). However, there was no difference of the membrane integrity at 0 h after thawing ($P > 0.05$).

Table 1 Means (± SEM) percentages of abnormal and normal cat spermatozoa in the ejaculate

Head abnormalities	
Proximal droplet	3.8±1.9
Distal droplet	1.7±1.4
Simple bent tail	12.2±6.9
Coil tail	4.4±3.7
Abnormal midpieces	1.0±0.5
Acrosome defect	2.1±1.9
Loose	3.3±1.9
Normal head	72.9±8.7
Midpiece and tail abnormalities	
Narrow	5.3±3.2
Narrow at the base	3.6±2.1
Pear-shape	1.5±0.9
Giant, broad,round	4.5±3.6
Abnormal contour	3.5±1.9
Abaxial	2.6±1.7
Loose	5.0±4.1
Normal midpiece and tail	75.5±7.5

N = Six cats with six ejaculates each.

Table 2 Means (± SEM) percentages of motility of cat spermatozoa at 0, 2, 4, and 6 h after thawing

Thawing method	0 h	2 h	4 h	6 h
At 37°C	59.7±9.7[a]	52.5±9.7[a]	38.1±10.9[a]	25.3±10.0[a]
At 37°C, diluted in Tris buffered	64.2±10.4[b]	61.7±9.3[b]	47.2±11.1[b]	33.1±13.7[b]
At 70°C	59.2±11.1[a]	53.3±9.9[a]	43.8±11.2[b]	26.7±10.7[a]
At 70°C, diluted in Tris buffered	65.4±11.2[c]	61.7±9.6[b]	51.1±12.6[b]	33.6±11.5[b]

Means within columns with different letters differ significantly ($P < 0.05$).

Table 3 Means (± SEM) progressive motility of cat spermatozoa at 0, 2, 4, and 6 h after thawing

Thawing method	0 h	2 h	4 h	6 h
At 37°C	3.6±0.6[a]	3.3±0.5[a]	2.5±0.7[a]	2.1±0.7[a]
At 37°C, diluted in Tris buffered	3.9±0.5[b]	3.7±0.5[b]	3.1±0.6[b]	2.4±0.9[b]
At 70°C	3.6±0.6[a]	3.4±0.5[a]	2.7±0.5[a]	2.0±0.5[a]
At 70°C, diluted in Tris buffered	4.1±0.4[b]	3.8±0.5[b]	3.3±0.6[b]	2.6±0.7[c]

Means within columns with different letters differ significantly ($P < 0.05$).

Table 4 Means (± SEM) percentages of intact membranes of cat spermatozoa at 0, 2, 4, and 6 h after thawing

Thawing method	0 h	2 h	4 h	6 h
At 37°C	61.0±12.5[a]	57.4±13.4[a]	48.3±10.7[a]	46.5±10.6[a,b]
At 37°C, diluted in Tris buffered	63.8±8.8[a]	62.9±9.1[b]	55.6±10.5[b]	51.5±10.2[a]
At 70°C	64.5±14.0[a]	60.2±12.5[a,b]	51.7±11.1[a,b]	44.8±13.8[b]
At 70°C, diluted in Tris buffered	65.1±14.7[a]	61.0±14.3[a,b]	55.7±14.3[b]	50.6±14.3[a]

Means within columns with different letters differ significantly ($P < 0.05$).

Table 5 Means (± SEM) percentages of cat spermatozoa with intact acrosomes at 0, 2, 4, and 6 h after thawing

Thawing method	0 h	2 h	4 h	6 h
At 37°C	39.7±19.0[a]	32.0±17.7[a]	28.2±17.4[a]	18.7±12.9[a]
At 37°C, diluted in Tris buffered	47.0±20.3[b]	39.7±18.6[b]	34.2±14.8[a]	23.7±13.2[a]
At 70°C	39.6±22.1[a]	31.3±19.3[a]	28.0±17.8[a]	17.8±13.0[a]
At 70°C, diluted in Tris buffered	47.7±20.2[a,b]	34.6±16.2[a,b]	32.3±16.0[a]	24.0±13.4[a]

Means within columns with different letters differ significantly ($P < 0.05$).

Discussion

With the cryoprotocol used in this study, frozen ejaculated cat spermatozoa can be thawed either at 37 or 70 °C, resulting in similar post-thaw sperm characteristics. However, thawing at 70°C for 6 s and diluted with Tris buffered resulted in the best motility at 0 h compared to the others. This is in agreement with the study in dogs, demonstrating that thawing at 70 °C is preferable for sperm longevity during incubation at 38 °C regardless of slow or fast freezing rates (Peña and Linde-Forsberg, 2000). Different rates of warming have been shown to influence post-thaw motility and acrosome integrity of dog spermatozoa (Olar et al., 1989, England, 1992). At rewarming, if not fast enough, the small ice crystals formed in the cell can recrystallize, building up larger crystals that will mechanically damage the cell. A too fast thawing when the freezing rate has been very slow, in contrast, might create a sudden osmotic stress to the dehydrated cell, resulting in swelling of the spermatozoa (Mazur 1984).

The thermoresistance test has been shown to be an accurate clinical test for predicting fresh human sperm fertilizing potential and the fertilization rate in *in vitro* fertilization (Alvarez et al., 1996). In boars, the thermoresistance test gives a better indication of fertility than does estimation of immediate post-thaw motility (Larsson and Einarsson, 1976). The results from this study indicated that neither thawing temperature nor dilution did influence the thermoresistance of cat ejaculated spermatozoa, indicated by the similar decreased values of sperm characteristics between different temperatures at thawing in a time-dependent manner.

Addition of Equex STM paste to the freezing extender has been postulated as causing reduction of sperm longevity during post-thaw *in vitro* incubation of cat epididymal spermatozoa (Axnér et al., 2004). Using the same cryoprotocol, lower reductions of all sperm characteristics during post-thaw *in vitro* incubation were demonstrated in this study compared to the previous report (Axnér et al., 2004), indicating that the ejaculated cat spermatozoa were more resistant to *in vitro* incubation in the medium containing Equex. Ejaculated and epididymal spermatozoa have different membrane characteristics. Ejaculated and epididymal spermatozoa have for example different susceptibility for cold shock (White, 1993). Since cat spermatozoa are easily damaged by the freeze-thaw process (Pope et al., 1991; Wood et al., 1993; Lengwinat and Blottener, 1994), the cryopretective effect of Equex on the sperm quality may be valuable for post-thaw ejaculated spermatozoa.

Significant volume change occurs at thawing when the cell exchanges intracellular cryoprotectant for re-entry of water (Gao et al., 1995). The better post-thaw results in the diluted samples in this study likely indicated that post-thaw dilution reduces osmotic stress caused by rewarmed cryoprotectants. However, it seems that membrane integrity was less sensitive to changes in osmolality than motility and acrosome integrity because similar percentages of membrane integrity were observed in post-thaw samples with and without a dilution. This is in agreement with the previous report in cat spermatozoa exposed to anisotonic conditions and return to isotonicity (Pukazhenthi et al., 2002).

In conclusions, Equex STM paste can be used successfully as a cryoprotectant for ejaculated cat spermatozoa without deleterious sperm longevity. With the freezing protocol used in this study, it is preferable to thaw frozen ejaculated cat spermatozoa at 70 °C for 6 s. A post-thaw dilution with Tris buffered solution is favorable to sperm motility and acrosome integrity.

Acknowledgement

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Output ที่ได้จากโครงการวิจัยที่ได้รับทุน

1. **ผลงานวิจัยส่งตีพิมพ์ในวารสารวิชาการระดับนานาชาติ 3 เรื่อง**
   1.1. Chatdarong, K., Manee-In, S., Thuwanut, P., Axnér, E and Lohachit, C. Effect of post-thaw dilution and thawing temperature on viability of cat spermatozoa. (in preparation).
   1.2. Chatdarong, K., Axnér, E., Lohachit, C. and Linde Forsberg, C. The optimal time and site for artificial insemination in the domestic cat. (in preparation).
   1.3. Thongphagdee, A., Numchaisrikha, P., Rungsiwiwut, R., Omsongkram, S., Chatdarong, K., Kamolnorranath, S. and Techakumphu, M. 2006. In vitro development of marbled cat embryos derived from interspecies somatic cell nuclear transfer. *Reprod. Dom. Anim.* 41, 219-226.
2. **ผลงานวิจัยนำเสนอในการประชุมวิชาการระดับนานาชาติ 4 เรื่อง**
   2.1. Axnér, E., Chatdarong, K., Lohachit, C. and Linde Forsberg, C. 2005. Effect of post-thaw dilution and thawing temperature on viability of cat spermatozoa. *Reprod. Dom. Anim.* 40, 359 (Abstract).
   2.2. Thongphagdee, A., Numchaisrika, P., Chatdarong, K., Wongtawan, T., Rungarunlert, S., Rattanakorn, P., Kamolnorranath, S., Dumnui, S. and Techakumphu, M. 2005. Cloned flat-headed cat (*Prionailurus planiceps*) embryos produced from interspecies somatic cell nuclear transfer. *Proceedings of the 1th Scientific Meeting of the Asian Zoo & Wildlife Medicine*, October 28-30th, Bangkok, 37-38 (Abstract).
   2.3. Chatdarong, K., Axnér, E., Lohachit, C. and Linde Forsberg, C. 2006. The optimal time and site for artificial insemination in the domestic cat. *Proceedings of the 5th biannual congress of European Veterinary Society for Small Animal Reproduction,* April 7-9th, Budapest, 310 (Abstract).
   2.4. Tharasanit, T., Manee-In S., Rungarunlert S., Sananmuang T., Chatdarong K., Techakamphu M. and Lohachit C. 2006. Effects of recombinant human follicle stimulating hormone on developmental competence of cat oocytes. *Proceedings of the 3rd annual conference of Asian Reproductive Biotechnology Society,* Nov.29th-Dec.3rd, Hanoi (Abstract)

3. **การนำผลงานวิจัยไปใช้ประโยชน์**

เชิงอนุรักษ์ สามารถนำไปใช้ในสัตว์ป่าตระกูลแมวที่หายากหรือใกล้สูญพันธุ์ โดยจัดตั้งศูนย์เก็บน้ำเชื้อแช่แข็งแมวป่าชนิดต่าง ๆ ที่มีอยู่ในสวนสัตว์ เพื่อนำไปใช้ในการผสมเทียม หรือเทคโนโลยีชีวภาพทางการสืบพันธุ์ขั้นสูงต่อไป เช่น การโคลนนิ่ง การปฏิสนธิในหลอดทดลอง เป็นต้น

เชิงวิชาการ งานวิจัยนี้ก่อให้เกิดการวิจัยต่อเนื่อง โดยนำตัวอสุจิแช่แข็งไปใช้ประโยชน์ในงานวิจัยที่ใช้เทคโนโลยีชีวภาพขั้นสูง และยังเป็นประโยชน์ทางการเรียนการสอนสำหรับนิสิตสัตวแพทย์

เชิงสาธารณะ งานวิจัยนี้ได้พัฒนาวิธีการแช่แข็งน้ำเชื้อแมว ซึ่งเป็นการรองรับการขยายตัวของการเลี้ยงแมวโดยเฉพาะแมวพันธุ์แท้เป็นสัตว์เลี้ยงกันมากขึ้น อีกทั้งยังเป็นการอนุรักษ์พันธุกรรมเพศผู้ของแมวไทยพันธุ์แท้ที่หายากในปัจจุบันได้อีกด้วย

ภาคผนวก

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In Vitro Development of Marbled Cat Embryos Derived from Interspecies Somatic Cell Nuclear Transfer

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Contents

The objective of the study was to investigate interspecies Somatic Cell Nuclear Transfer (iSCNT) techniques in marbled cats (*Pardofelis marmorata*), using domestic cat and rabbit oocytes as the recipient cytoplasm. The recipient oocytes were obtained from ovariohysterectomized cats and superovulated rabbits. The donor cells were collected from a male marbled cat that had died in captivity. Experiment 1 was conducted to observe the development of cloned marbled cat embryos (marbled cat donor cells-domestic cat oocytes; MC-DC), derived from oocytes matured for 24, 36 and 42 h. The result showed that the developmental rates of MC-DC cloned embryos at the 4–8 cell and the morula stages derived from oocytes cultured for 24 h were significantly greater than those cultured for 36 and 42 h ($p < 0.05$). Experiment 2 was conducted to compare the fusion rate of MC-DC couplets, fused by inducing different fusion voltages, 2.1 or 2.4 kV/cm. The result showed that there was no difference in fusion efficiency between the 2.1 and 2.4 kV/cm fusion protocols. Experiment 3 was conducted to compare the developmental rate of MC-DC and domestic cat (DC-DC) cloned embryos. *In vitro* fertilized cat embryos served as a control. The development of MC-DC and DC-DC cloned embryos to the 4- to 8-cell, morula and blastocyst stages was not significantly different. However, the development rates at morula and blastocyst stages of control were significantly greater than those of cloned embryos ($p < 0.05$). Experiment 4 rabbit (RB) oocytes were used as a recipient cytoplasm for marbled cat and domestic cat cloned embryos (MC- RB and DC-RB). RB-RB cloned embryos served as a control. There were no differences in the developmental rates between MC-RB, DC-RB and RB-RB embryos. In conclusion, marbled cat fibroblast cells can be reprogrammed in domestic cat and rabbit oocytes, and by using iSCNT it might be possible to produce marbled cat offspring in the future.

Introduction

Felis marmorata or *Pardofelis marmorata* (marbled cat), one of the small wild cats previously found in the north-east of India through to the south-east of Asia, to Borneo and Sumatra, is considered to be nearly extinct, according to CITES appendix I. Wildlife conservation strategies including Assisted Reproductive Technologies (ARTs) are needed for the maintenance of its population. Somatic Cell Nuclear Transfer (SCNT) is part of ARTs, which supports captive breeding programmes for selected wildlife (Pope 2000) and is regarded as being beneficial for sustaining genetic biodiversity (Holt and Pickard 1999).

Interspecies SCNT (iSCNT) has become established due to a lack of oocytes from the wild to produce cloned offspring. Accordingly, host oocytes from both close-related and unrelated animals are preferred for iSCNT studies. Domestic cat oocytes collected after routine ovariohysterectomy are normally used for endangered felid cloning. The use of rabbit oocytes has been shown in many reports to enable the production of giant panda (Chen et al. 2002; Li et al. 2002), bovine (Techakumphu et al. 2005), elephant (Numchaisrika et al. 2005), human (Chen et al. 2003) and cat (Wen et al. 2003) cloned embryos. In addition, many attempts have been made to observe the capacity of bovine oocytes to reprogramme the donor cells of several species including rats, sheep, pigs (Dominko et al. 1999), buffaloes (Kitiyanant et al. 2001), gaurs (Lanza et al. 2000; Hammer et al. 2001), monkeys (Dominko et al. 1999; Simerly et al. 2004), chickens (Kim et al., 2004), whales (Ikumi et al. 2004) and humans (Chang et al. 2003).

The success of offspring production with iSCNT has been shown in four endangered species: gaurs (Lanza et al. 2000), mouflons (Loi et al. 2001), bantengs (Janssen et al. 2004) and African wild cats (Gomez et al. 2004). In felids species, cloned domestic cat embryos have been successfully produced as a model for wild felids (Shin et al. 2002; Skrzyszowska et al. 2002; Gomez et al. 2003, 2004; Kitiyanant et al. 2003; Wen et al. 2003; Yin et al. 2005). Leopard cat (Lorthongpanich et al. 2004) and African wild cat (Gomez et al. 2004) embryos have also been produced from enucleated domestic cat oocytes. The birth of cloned domestic cats (Shin et al. 2002; Yin et al. 2005) and African wild cats exhibits the effectiveness of iSCNT.

The present studies were conducted to observe the development of cloned marbled cat embryos derived from domestic cat oocytes (MC-DC) cultured for 24, 36 and 42 h (experiment 1), to compare the fusion rate of MC-DC couplets fused by inducing different fusion voltages (experiment 2), to compare the developmental rate of MC-DC and domestic cat (DC-DC) cloned embryos (experiment 3), and to observe the capacity of the recipient cytoplasm, rabbit oocytes (RB), to reprogramme marbled and domestic cat fibroblast cells (MC- and DC-RB) (experiment 4).

Materials and Methods

All chemicals were purchased from Sigma Co. (St Louis, MO, USA) unless otherwise stated. Media were prepared weekly, filtered (0.2 μ, no. 16534 Sartorius, Minisart) and kept in sterile tubes. The bicarbonate-buffered cultured media were incubated at 38.5°C under 5% CO_2 in air at least 4 h before use.

Preparation of recipient cytoplasm

Domestic cat oocytes

Domestic cat ovaries were obtained after ovariohysterectomy and stored at room temperature in phosphate buffer saline (PBS; Gibco, Grand Island, NY, USA), supplemented with 5% foetal calf serum (FCS; Gibco), and 10 IU/ml penicillin and streptomycin (Gibco). The oocytes were collected within 4 h after the ovaries were removed by mincing the ovaries in an oocyte collecting medium, which was composed of Dulbecco's modified eagle medium (DMEM; Gibco) supplemented with 0.292 g/ml glutamine, 0.026 g/ml pyruvate, 0.4% bovine serum albumin (BSA fraction V), 100 IU penicillin, 100 μg/ml streptomycin and 10 mM HEPES buffer. Only oocytes with compact cumulus cells of more than two layers and homogenous dark ooplasm were selected. They were washed in the oocyte collecting medium, three times and once in an oocyte culture medium, which was composed of DMEM supplemented with 0.292 g/ml glutamine, 0.026 g/ml pyruvate, 0.4% BSA, 100 IU penicillin, 100 μg/ml streptomycin, 1 μg/ml porcine lutinizing hormone (LH), 1 μg/ml porcine follicle stimulating hormone (FSH) and 1 μg/ml oestradiol (Wood and Wildt 1997). Five to 10 oocytes were cultured in a 50 μl drop of oocyte culture medium, at 38.5°C, under 5% CO_2 in air, for 24, 36 and 42 h (experiment 1), and cultured for 24 h (experiments 2 and 3). The cumulus cells were removed from the oocytes by gentle pipetting in 0.1% hyaluronidase. Mature oocytes which were defined as presenting the first polar body and confirmed by 15 μg/ml Hoechst 33342 staining, were selected and enucleated in a handling medium; tissue culture medium 199 (TCM199) with HEPES buffer containing 7.5 μg/ml cytochalacin B (Fig. 1a). Enucleation was performed by aspirating the first polar body and metaphase II (M II) chromosomes with a small volume of surrounding cytoplasm which were located visually under UV light.

Rabbit oocytes

Oocytes were obtained from a superovulated New Zealand White rabbit doe which was given 21 mg FSH,100 IU hCG and then mated with a vasectomized male (Techakumphu et al. 2004). The mature oocytes (16 h post-coitus) were flushed from the oviducts using PBS solution. The cumulus cells were removed and oocytes were enucleated in a manner similar to that carried out for cat oocytes.

Preparation of donor nuclei

Muscle tissue from a dead marbled cat from Khao Kheow open zoo, a domestic cat and a rabbit were stored in modified eagle's medium (MEM) supplemented with 5% FCS, penicillin and streptomycin, at 4°C, within 12 h after collection. The tissues were washed in MEM, sliced into small pieces and cultured in a 30-mm Petri dish containing MEM supplemented with 10% FCS, penicillin and streptomycin, at 38.5°C, under 5% CO_2 in air. Fibroblast cells (Fig. 1b) were sub-cultured by washing with PBS, trypsinized by 0.25% trypsin and washed in MEM, supplemented with 5% FCS and centrifuged at 1000 × *g* for 5 min. Fibroblast cells were cultured in MEM, supplemented with 10% FCS, penicillin and streptomycin, in a 60-mm Petri dish, at 38.5°C, under 5% CO_2 in air. The confluent cells were frozen with 10% DMSO in FCS and stored in liquid nitrogen for future use. The frozen cells, between passages 4 and 10 of the culture, were thawed and used

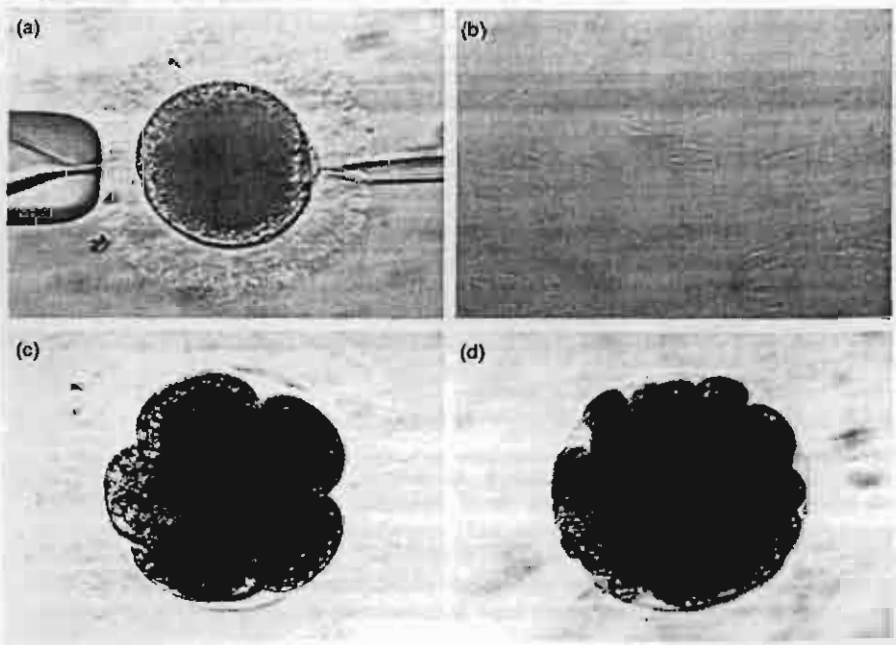

Fig. 1. Enucleation (cat oocyte) (a) (×200), marbled cat fibroblast cells (b) (×100), MC-DC cloned embryos at the 8-cell stage (c) and a compact morula (d) (×300)

as a source of donor nuclei. The cells were cultured as previously described, until reaching 60% confluency, then starved by culturing in MEM, supplemented with 0.5% FCS, at 38.5°C, under 5% CO_2 in air, for 1–5 days prior to the nuclear transfer procedure.

Nuclear transfer, fusion and activation

Domestic cat oocytes

Nuclear transfer (NT), fusion and activation methods were performed as described by Skrzyszowska et al. (2002). An individual donor cell was transferred into the perivitelline space of an enucleated oocyte. NT oocytes were washed and incubated in a fusion medium (0.3 M mannitol, 0.1 mM $CaCl_2$, 0.1 mM $MgSO_4$ and 0.05% fatty acid-free BSA) for 1 min. The NT oocytes were transferred to a fusion chamber between two platinum electrodes and overlaid with fusion medium. Then NT oocytes were induced by two direct current (DC) pulses of 2.0 kV/cm for 15 μs in experiment 1: 2.1 kV (Kitiyanant et al. 2003) or 2.4 kV (Gomez et al. 2003) for 80 μs in experiment 2 and 2.4 kV/cm for 80 μs in experiment 3. Thereafter, the couplets were cultured in B2 medium (INRA, CCD, Paris, France) supplemented with 10% FCS at 38.5°C, in 5% CO_2, for 2 h. The fused NT embryos were activated by exposing them to 7% ethanol for 5 min in experiment 1 or by inducing them with 2 DC pulses of 1.2 kV/cm for 40 μs in experiments 2 and 3. They were then cultured in B2 medium, supplemented with 10% FCS and 10 μg/ml cycloheximide, at 38.5°C, in 5% CO_2, for 4 h. In experiments 2 and 3, a synthetic oviductal fluid (SOF) (Wen et al. 2003) was used instead of B2 medium.

Rabbit oocytes

The procedure of NT was similar to that carried out for domestic cat oocytes, but the couplets were fused by inducing 3 DC pulses of 3.2 kV/cm for 20 μs in the fusion medium (Chesne et al. 2002). Fused oocytes were subsequently held in SOF and supplemented with 10% FCS for 1 h. For activation, the fused couplets were induced by using the same procedure of fusion and were then incubated in SOF, supplemented with 5 μg/ml cycloheximide and 2 mM 6-DMAP, for 1 h.

In vitro culture of embryos

The couplets were cultured in a 50-μl drop of SOF medium supplemented with 10% FCS, at 38.5°C, under 5% CO_2, for 7 days, but in experiment 1, they were cultured in B2 supplemented with 10% FCS for 2 days and then co-cultured with Vero cells until day 7.

In vitro fertilization

In vitro fertilization (IVF) served as a control in experiment 3. Semen was collected from an adult domestic cat using electro-ejaculation. The semen was frozen in 0.25 ml straws, as described by Axner et al. (2004). On the day of insemination, the frozen semen straw was thawed and the spermatozoa were prepared by the swim-up method, using swim up medium (M199, supplemented with 0.292 g/ml glutamine, 0.026 g/ml pyruvate, 0.4% BSA, 100 IU penicillin, 100 μg/ml streptomycin), at 38.5°C, for 15 min. The 4×10^4 spermatozoa were subsequently co-incubated together with five to 10 oocytes (cultured *in vitro* for 24 h), at 38.5°C, under 5% CO_2, in a 100 μl drop of IVF medium (swim up medium but the 0.4% BSA was changed to 0.6%). At 18 h post-insemination, the oocytes were washed in IVF medium and later cultured in SOF medium, supplemented with 10% FCS for 7 days.

Experimental design

Experiment 1

Fourteen replicates were carried out to observe the development of MC-DC cloned embryos derived from the cat oocytes, matured for 24, 36 and 42 h.

Experiment 2

Four replicates were conducted to compare the fusion and developmental capacity of MC-DC cloned embryos, derived by using different fusion voltages (2.1 and 2.4 kV/cm).

Experiment 3

Ten replicates were performed to compare the development of MC-DC and DC-DC cloned embryos. *In vitro* fertilized cat embryos served as a control.

Experiment 4

Four replicates were conducted to observe the development of MC-RB and DC-RB cloned embryos. RB-RB cloned embryos served as a control.

Statistical analyses

The fusion rate and developmental rate of embryos were compared according to the different culture periods (24, 36 and 42 h), fusion protocols (2.1 and 2.4 kV/cm), and donor cells (MC- and DC-DC and MC-, DC- and RB-RB), using chi-square analysis. Data with number of observation below 5 were analysed by Fisher's exact test. p-values ≤ 0.05 were considered statistically significant.

Results

Experiment 1: the *in vitro* development of cloned MC-DC embryos derived from 24, 36 and 42 h matured oocytes

The number of cloned embryos at the 2- to 4-cell stage derived from the oocytes cultured for 24, 36 and 42 h was not significantly different. However, the developmental rates to the 4–8 cell (Fig. 1c) and morula stages (Fig. 1d) of oocytes cultured for 24 h were greater than those cultured for 36 and 42 h (4- to 8-cell stage: 27.7%, 11.6% and 9.9%; and morula stage: 9.2%, 4.3% and 0%, respectively; $p < 0.05$; Table 1).

Table 1. The development of cloned marbled cat embryos derived from 24, 36 and 42 h matured cat oocytes

Culture period (h)	n	2–4, n (%)	>4–8, n (%)	morula, n (%)	blastocyst, n (%)
24	119	53 (44.5)	33 (27.7)[a]	11 (9.2)[a]	0
36	138	50 (36.2)	16 (11.6)[b]	6 (4.3)[b]	0
42	203	75 (37)	20 (9.9)[b]	0 (0)[c]	0

Values within a column with different letters differ ($p < 0.05$).

Experiment 2: the fusion and developmental capacity of cloned MC-DC embryos derived from different fusion voltages (2.1 and 2.4 kV/cm)

The fusion efficiency of couplets using the 2.1 and 2.4 kV/cm fusion protocols was not significantly different (46% vs 48.5%). The developmental rate of MC-DC embryos derived from the 2.4 kV/cm fusion protocol was not significantly different from those of the 2.1 kV/cm fusion protocol (Table 2).

Experiment 3: the development of MC- and DC-DC cloned embryos

The development of cloned MC- and DC-DC embryos to the 4–8 cell, morula and blastocyst stages (Fig. 2a,b) was not significantly different (4–8 cell: 56% vs 50%; the morula: 8% vs 8.3%; and blastocyst stages: 0% vs 4.2%, respectively). The development of IVF embryos reaching the morula and blastocyst stages was greater than those of cloned MC- and DC-DC embryos (Table 3).

Experiment 4: the development of MC-, DC- and RB-RB cloned embryos

The development of the cloned MC- (Fig. 3a–d), DC- (Fig. 4a,b) and RB-RB embryos to the 4- to 8-cell stage was 100%, 85% and 96.4%, at the morula stage 19.2%, 23.1% and 35.7% and at the blastocyst stage 11.5%, 7.7% and 14.3%, respectively; these were not significantly different (Table 4).

Table 2. The comparison of fusion and developmental capacity of cloned MC-DC embryos fused by different voltages

Voltage kV/cm	n	fused, n (%)	4–8, n (%)	morula, n (%)	blastocyst, n (%)
2.1	37	17 (46)	12 (70.6)	5 (29.4)	0
2.4	33	16 (48.5)	13 (81)	8 (50)	0

Table 3. The development of MC- and DC-DC cloned embryos

Donor cell-oocyte	n	fused, n (%)	4–8, n (%)	morula, n (%)	blastocyst, n (%)
MC-DC	63	25 (40)	14 (56)	2 (8)[a]	0[a]
DC-DC	60	24 (40)	12 (50)	2 (8.3)[a]	1 (4.2)[a]
IVF (control)	53	–	28 (52.8)	12 (22.6)[b]	5 (9.4)[b]

Values within a column with different letters differ ($p < 0.05$).

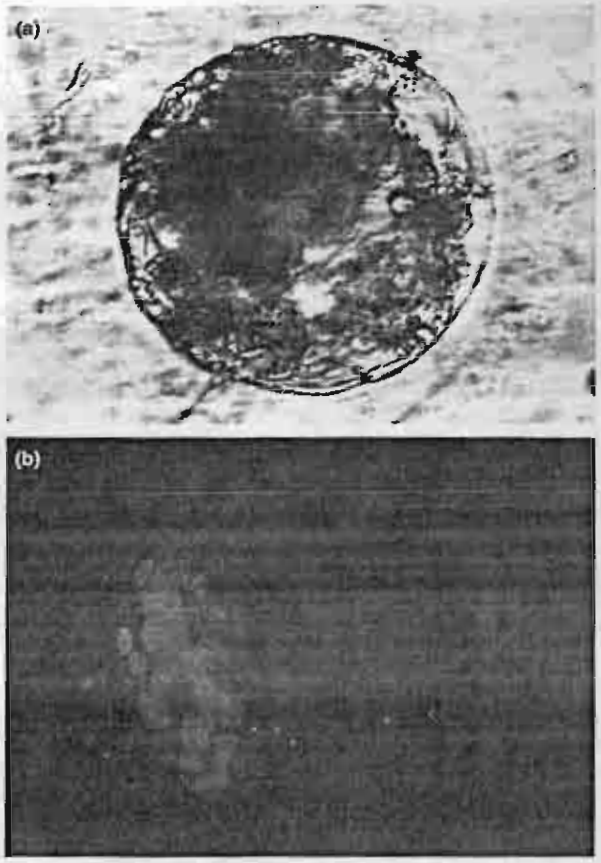

Fig. 2. A DC-DC cloned blastocyst (a) and a Hoechst stained blastocyst, under UV light (b) (×300)

Discussion

Currently, iSCNT has been applied in various species, however, low success rates of cloned embryo production is the major obstacle to a practical application. Many factors affect cloned embryo development, such as oocyte culture periods (Skrzyszowska et al. 2002), cell type, the stages of the donor cell cycle (Miyoshi et al. 2002) and the gender of the donor cell lines (Gomez et al. 2003; Sansinena et al. 2005).

The oocyte maturation process is one of the crucial steps for the subsequent development of cloned embryos. In experiment 1, the developmental rate of cloned MC-DC embryos derived from oocytes matured for 24 h was greater than those matured for 36 and 42 h. These indicated that domestic cat oocytes cultured for 24 h were more able to develop to the 8-cell stage than those cultured longer. The time of maturation has an influence on the level of the maturation promoting factor (MPF) and the mitogen activated protein kinases (MAPK). These are necessary for initiating germinal vesicle breakdown, meiotic progression, the arrest of oocyte development at the MII stage, which subsequently allows donor chromosomes to condense properly and enhance correct ploidy (Hayes et al., 2005). Bogliolo et al. (2004) found that after 24 h of incubation, matured oocytes had a higher MPF and

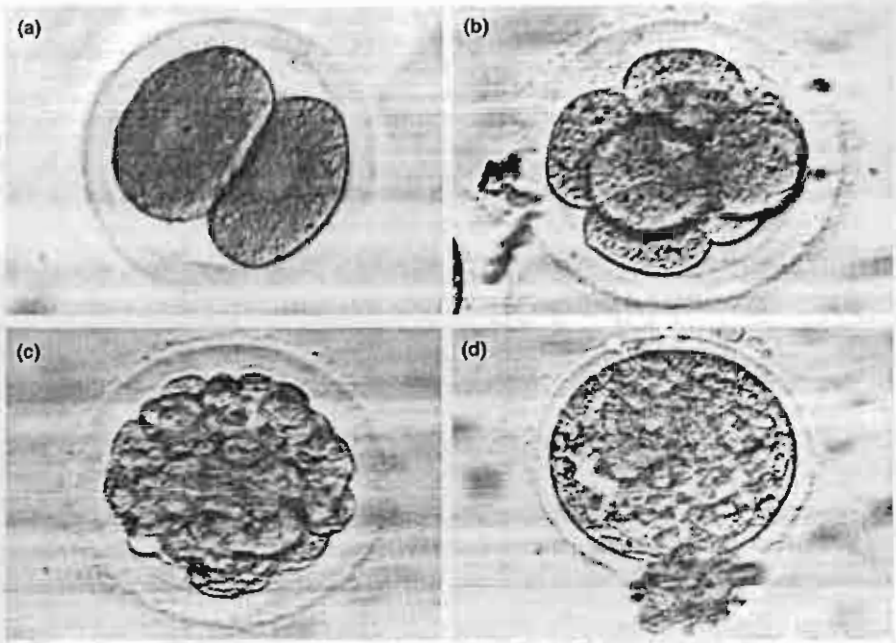

Fig. 3. MC-RB cloned embryos at different stages: 2-cell stage (a), 8-cell stage (b), compact morula (c), hatching blastocyst (d) (×300)

MAPK levels than those cultured for 40 h. Accordingly, lower levels of MPF and MAPK may cause less development and incomplete reprogramming of the embryos, as found in the 36 and 42 h matured oocytes. Our findings are in agreement with Skrzyszowska et al. (2002), who showed that a prolonged culture period for cat oocytes of over 40 h decreased the developmental competence of the reconstituted embryos. In cattle, Miyoshi et al. (2002) suggested using rapidly matured oocytes, which would represent a novel way to improve the developmental rates of cloned offspring. The oocyte culture period had a slight effect on the maturation rate in our study. The oocyte culture periods 24, 36 and 42 h gave similar maturation rates (52%, 53% and 56%; data not shown), which corresponded to previous reports (Farstad 2000; Rungsiwiwut et al. 2005). However, many cat oocytes reaching the MII stage (66–70%) were obtained from the oocytes cultured for 42–45 h (Katska-Ksiazkiewicz et al. 2003). In this study, oocytes cultured for 42 h revealed deteriorating characteristics, such as aging-like fragmentation of the polar body, debris in the perivitelline space and clumping of the chromosomes at the metaphase plate, similar to that previously reported (Skrzyszowska et al. 2002; Katska-Ksiazkiewicz et al. 2003). This supported the finding that the prolonged culture of oocytes does not make them suitable as recipient cytoplasm for nuclear transfer.

This study found that inducing 2 DC pulses of either 2.1 or 2.4 kV/cm for 80 μs gave a similar fusion rate. The difference between both electrical pulse voltages (0.3 kV/cm) may not be significantly different in the MC-DC fusion process. Fusion is an important process, the inducing electrical pulses cause a reversible physical breakdown of bi-lipid membranes, resulting in the formation of temporary pores. When juxtapositions pores in the membranes of two cells reseal, following the DC pulses, the cells may fuse. Our fusion rate was slightly higher than the report by Wen et al. (2003) (42.7%), because the electrical voltage was greater than the 1.4 kV/cm used by them. Fusion efficiency was better (60–66%) when the number of inducing pulses was increased to 4 (Kitiyanant et al. 2003). Moreover, the addition of an alternating current (AC) pulse before the inducing DC pulses was beneficial for fusion and resulted in a fusion rate of 73–87% (Gomez et al. 2003). AC pulses seem to assure a better alignment of the recipient cytoplast and donor cells.

Although the development of interspecies MC-DC and intraspecies DC-DC cloned embryos to the morula stage was similar in our study, only DC nuclei had the ability to develop to the blastocyst stage in domestic cat oocytes. It seemed to be a species-specific response between the donor nucleus and the recipient cytoplasm in this study. In contrast, Gomez et al. (2003) demonstrated that African wild cat fibroblasts can be differentiated at greater rates than domestic cat fibroblasts, in enucleated domestic cat oocytes. The limitation of cloned MC- and DC-DC production is not only due to incomplete reprogramming but also due to embryonic blocking causing a low embryo developmental rate. Moreover, the fragmentation of cloned embryos that fails to undergo chromatin remodelling, occurred in 18% (Gomez et al. 2003). As with cat embryos cultured *in vitro*, the first embryonic arrest happened at the 5- to 8-cell stage, which corresponded to the transition period from maternal to embryonic control and the second arrest happened between the morula and blastocyst stages (Kanda et al. 1995). The *in vitro* fertilized cat embryos served as a control for evaluating the ability of the oocytes to fertilize and subsequently cleave *in vitro*. These results suggest that these oocytes could be fertilized and developed to the morula and blastocyst stages.

Table 4. The development of marbled cat and domestic cat embryos produced from rabbit oocytes

Donor cell-oocyte	n	fused, n (%)	4–8, n (%)	morula, n (%)	blastocyst, n (%)
MC-RB	56	26 (46.4)	26 (100)	5 (19.2)	3 (11.5)
DC-RB	53	26 (49.1)	22 (85)	6 (23.1)	2 (7.7)
RB-RB	55	28 (51)	27 (96.4)	10 (35.7)	4 (14.3)

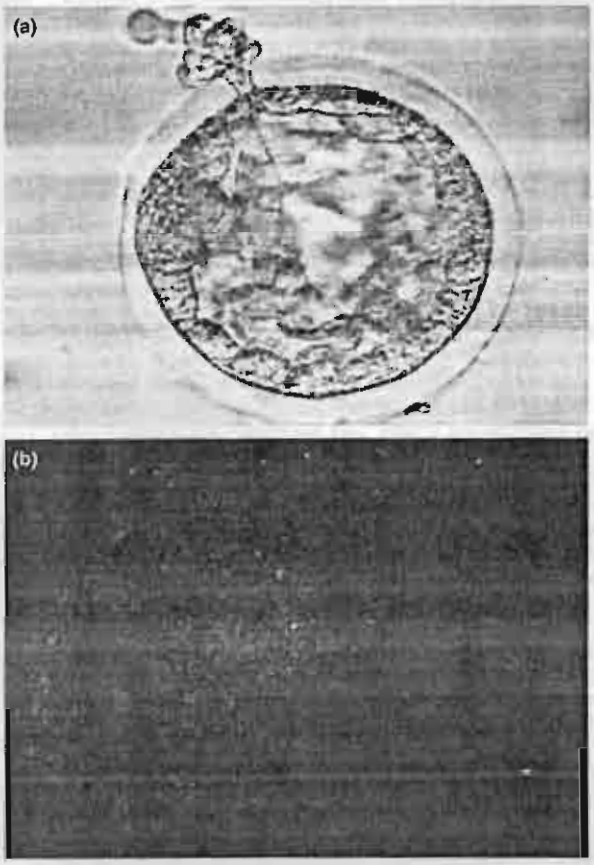

Fig. 4. A DC-RB cloned blastocyst under an inverted microscope (a) (×300) and a Hoechst stained hatching blastocyst, under UV light (b) (×100)

Interestingly, when rabbit oocytes served as the recipient cytoplasm, receiving DC or MC donor nuclei, the cloned MC- and DC-RB embryos overcame the morula to blastocyst blocking. The development of interspecies cloned embryos, MC- and DC-RB, was not significantly different from intraspecies cloned embryos, RB-RB. This study suggests that rabbit oocytes served as a valuable recipient cytoplasm for nuclear transfer for both marbled and domestic cats. The advantages of using rabbit oocytes as the recipient cytoplasm for interspecies cloning are an effective superovulation programme (Techakumphu et al. 2004) and a greater number of matured oocytes (10–40 oocytes) that can be collected from each rabbit (data not shown). Producing cloned embryos or embryonic stem cells from such a species is also useful because oocytes are difficult to obtain from others and because of possible ethical laws (Chang et al. 2003; Chen et al. 2003).

Despite this, iSCNT is still in the developmental stage and in-depth understanding of reprogramming and embryo development has not been completely clarified. The role and the effect of heteroplasmy, the presence of donor nuclei and the recipient oocyte cytoplasm (including mitochondrial DNA and other organelles), on the success of cloned embryo production has not been well documented. It has been reported that mitochondrial DNA recombination is likely to affect SCNT (Hiendleder et al. 2005). Before implantation, Wen et al. (2003) found that mitochondria from both panda fibroblasts and rabbit oocytes coexisted. However, after implantation, mitochondria from the donor panda cells were detectable and those from the recipient rabbit oocytes were eliminated. An incompatibility between the cytoplasm of donor cells and the recipient cytoplasm may influence the development of cloned embryos in the early stages as well as during implantation. Our study did not investigate the heteroplasmy issue, however, we did not find any differences in the development of the early stages of inter-and intraspecies (control) embryos, which may imply that the effects of heteroplasmy on the development of MC-DC, MC-RB and DC-RB are not evident during early stages.

In conclusion, marbled cat fibroblast cells can be reprogrammed in domestic cat and rabbit oocytes, and by using iSCNT it might be possible to produce marbled cat offspring in the future.

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Reprod Dom Anim 40, 345-410

Abstract P63

The Effect of Average Daily Gain of Pubertal Holstein Heifers on Progesterone and cholesterol Patterns During the Estrous Cycle

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Fertility of heifers is dependent on nutrition during the growing period, and the fertility of cows is related to progesterone and cholesterol patterns during the estrous cycle. The present experiment investigated the effects of 3 different average daily gains (ADG) imposed for 3 months on progesterone secretion during the estrous cycle and cholesterol patterns. Thirty pubertal Holstein heifers were allocated according to age (14–16 months), weight (302 456 kg) and body condition score (BCS: 2.0 to 3.3; scale 1 5) to 3 treatment groups. A balanced diet was fed with the target of 1.0 (high; H), 0.8 (reference; R) and 0.6 (low; L) kg d ADG respectively. The animals were weighed every two weeks and the amount of feed adjusted accordingly. During the 4th month of treatment, blood samples were obtained twice a day (0700 and 1600 h) during a whole estrous cycle to assay progesterone and cholesterol concentrations. At the end of the sampling period the observed ADG were 1.10 ± 0.27, 0.76 ± 0.21, 0.60 ± 0.26 kg/d (mean ± sd) and BCS 3.16 ± 0.17, 2.39 ± 0.02, 2.19 ± 0.2 for H, R and L experimental groups, respectively. Duration of the estrous cycle (H:21.2 ± 1.1;R:21.2 ± 1.7; L:21.7 ± 1.9 days), area under the curve of progesterone values (H:189.7 ± 41.02; R:201.28 ± 44.79; L: 210.36 ± 51.47 arbitrary units) and of cholesterol (H: 49.4 ± 7.38; R:44.15 ± 7.58; L:46.28 ± 7.85 arbitrary units) were not different between treatment groups. Therefore, within the present experimental conditions, short variations in pubertal heifer diets did not affect progesterone nor cholesterol concentrations.

Abstract P64

Influence of Pre- and Postpartum Energy Status on the Resumption of Ovulation in Postpartum Limousin Suckled Beef Cows

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Whereas there is agreement that prepartum nutrition affects the interval to1st ovulation in suckling cows, the effects of postpartum nutrition on reproduction are inconsistent. Moreover, recent data suggest that individual energy status could be a more important factor than the energy level of the diet in determining the effect of nutrition on reproduction. Accordingly, relationships between pre- and postpartum energy status and time to resumption of ovulation in Limousin beef cows was evaluated under field conditions in animals fed to their specific requirements. Over 3 years, 138 calving periods (26 primiparous, 112 multiparous cows) were monitored for 8 weeks prepartum until the 1st postpartum ovulation. Energy status was evaluated from the concentrations of NEFA in blood samples collected weekly; occurrence of cyclicity was determined by progesterone assay. The first postpartum luteal phase was recorded 9.9 ± 2.0 and 7.7 + 1.4 weeks postpartum for primi- and multiparous cows respectively ($p < 0.01$).In primiparous cows, high concentrations of NEFA throughout the last 4 weeks of pregnancy as well as during the first 2 weeks of lactation were related to delayed resumption of ovulation. In multiparous cows, neither prepartum nor postpartum NEFA concentrations were related to time of ovulation. In conclusion, resumption of ovulation is more dependent on energy status in primiparous than in multiparous Limousin cows and is related to pre- as well as postpartum situation. Recommendation for nutrition of primiparous cows during the peripartum period should be re-evaluated.

Abstract P65

Effect of Feeding Red Clover Silage Containing Phyto-Oestrogens on Plasma Progesterone and Fertility of Ewes

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In Finland, the main source of phyto-oestrogens (p-E) for ruminants is red clover. The effect of red clover silage on blood p-E and progesterone (P_4) concentrations and on fertility was studied in 20 nulliparous Finnish Landrace ewes in two equal groups, fed either red clover or timothy-fescue grass silage. After 9 w. rams were introduced to the flocks for 8 w, then plasma for P_4 analysis was collected once weekly for 6 w. After this, the ewes were slaughtered and genital organs examined. Feed and serum samples collected once monthly were analysed with liquid chromatography for contents of p-E. In addition, metabolites of p-E common in ruminants were analysed in serum. Red clover silage contained on average 0.07% daidzein, 0.05% genistein, 0.60% formononetin and 0.35% biochanin-A in dry matter and serum of ewes fed with red clover formononetin 0.06 μg·ml and equol 5.6 μg·ml. No p-Es were found in grass silage or in serum of grass silage fed ewes. All animals conceived, 2.2 foetuses per ewe, were found in grass group and 2.1 per ewe, in red clover group. The average plasma P_4 rose gradually from 16 to 24 nmol·l and from 24 to 34 nmol·l from 7th to 12th week of pregnancy in red clover and grass groups, respectively. P_4 levels were significantly lower in red clover group ($p < 0.001$). Red clover feed increased concentrations of p-E metabolite, equol, and lowered P_4 concentration in blood. However, no effects on fertility, conception rate or number of foetuses, were detected.

Abstract P66

Effect of Post-Thaw Dilution and Thawing Temperature on Viability of Epididymal Cat Spermatozoa

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Equex STM paste has a protective effect on the acrosomes of feline epididymal spermatozoa during freezing but a toxic effect during post-thaw incubation at 37°C (Axnér et al. Anim Reprod Sci. 84, 179–191; 2004). The objective was to evaluate if post thaw dilution or thawing temperature would affect quality and longevity of ejaculated cat spermatozoa. Nine male cats were subjected to electroejaculation. The semen was frozen in 0.25 ml straws (Axnér et al. 2004). Straws from each ejaculate were thawed 1) in 0.25 ml Tris at 37°C for 15 s, 2) without dilution at 37°C for 15 s 3) in 0.25 ml Tris at 70°C for 6 s, 4) without dilution at 70°C for 6 s. Sperm motility and membrane integrity (SYBR-14/EthD-1) were evaluated after collection and at times 0, 2, 4 and 6 hours post-thaw (Axnér et al. 2004). The different treatments were analysed with GLM or a Friedman's test and pairwise comparisons were made with a paired t-test or a Wilcoxon one-sample test. At time 0 mean motility ranged between 62.8 ± 13.0% and 74.4 ± 6.8% and mean membrane integrity between 67.4 ± 9.21% and 74.1 ± 5.1% for the different treatments with no significant difference between treatments. At 6 hours membrane integrity was significantly better for treatment 1 than 2 (41.3 ± 15.7 vs. 28.3 ± 8.5 $p < 0.05$) and for treatment 3 than 4 (41.7 ± 18.1 vs 29.0 ± 12.4 $p > 0.05$). Motility did not differ significantly between treatments. In conclusion, post-thaw dilution of cryopreserved cat semen has a beneficial effect on post-thaw sperm longevity.

Abstract P67

Metabolism of IVP Calves Born by Caesarean Section or by Vaginal Delivery

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Calves born after *in vitro* production (IVP) may have decreased neonatal viability. A number of IVP calves are delivered by caesarean

Burmese python (*Python molurus bivittatus*) in snake farms have shown signs of swelling under mandibular region, dense mass and have bloody fluid. The isolation of bacteria showed *Pseudomonas spp.* and *Aeromonas spp.*

Intermandibular cellulites or Pharyngeal phlegmon are infectious diseases in various species of snakes. Cellulitesis is an acute, diffuse, spreading, edematous, suppurative inflammation of the deep subcutaneous tissues and sometimes muscle. Sometimes there is formation of abscess and the skin is warm and tender. It is usually caused by infection of a wound, burn, or other cutaneous lesion by bacteria, especially group A streptococci swelling and necrosis of the wall of the pharynx in with toxemia and respiratory distress; it may be fatal. In most snakes and pythons intermandibular cellulites are caused by the synergistic effects of *Pseudomonas fluorescens* and *Aeromonas hydrophila* infection.

The progress of intermandibular cellulites status in pythons is followed by mouth rot or infectious stomatitis. This condition is fatal within a short period. This condition requires aggressive therapy which includes potent bacteriocidal antibiotics, and supportive physiological fluid replacement. This infectious condition has responded well to aminoglycoside antibiotic especially butirosin sulphate.

No. 42: A CASE REPORT: VASECTOMY IN A MALE ROYAL BENGAL TIGER

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A male Royal Bengal Tiger 5 year old was living with a group of show tigers in the same cage. Afterwards we found out that he had been bred with a female tiger in that cage. The female tiger got pregnant and gave birth to 3 cubs. All the cubs were injured by the other tigers in the same cage and eventually died. We had to protect the tigers. So we conducted a vasectomy to stop breeding. can do their own job in the next day.

No. 43: CLONED FLAT-HEADED CAT (*Prionailurus planiceps*) EMBRYOS PRODUCED FROM INTERSPECIES SOMATIC CELL NUCLEAR TRANSFER

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Flat-headed cats (*Prionailurus planiceps*; FC), one of the small wild cats of Southeast Asia, are considered to be extremely endangered. Recently, two female FC were rediscovered in Thailand. Interspecies somatic cell nuclear transfer (ISCNT), using FC fibroblast cells as a donor cell and domestic cat oocytes as a recipient cytoplasm, may be a useful means, of producing FC embryos for embryology study and offspring production.

The objective of the study was to investigate the development of the FC embryos produced from iSCNT technique. Immature oocytes were collected from ovaries of ovariohysterectomized domestic cats, and cultured *in vitro* in TCM 199 at 38.5° C, under 5% CO_2, in air, for 24 h. Metaphase II plate and the first polar body of matured oocytes, which were located by Hoechst staining, under UV light, were removed. The FC donor cells (passage 2-4) were inserted into perivitelline spaces of enucleated oocytes. The couplets were fused by inducing 3 DC pulses of 2.4 kV/cm for 50 µs. The fused couplets were activated by inducing 3 DC pulses of 1.2 kV/cm for 50 µs, and were incubated in SOF medium, supplemented with 10% FCS, cycloheximide and cytochalacin B, for 4 h. Thereafter, the couplets were cultured in SOF, supplemented with 5% FCS, at 38.5° C, under 5% CO_2, in air. The fusion rate of the couplets was 79% (60/76). The developmental rates of cloned FC embryos (embryos/fused couplets) at 2-4 cell, 4-8 cell, 8-16 cell, morula and blastocyst stages were 97%, 83%, 65%, 53% and 8%, respectively. The study indicates the first success in producing FC embryos using iSCNT technique, which will be useful for producing offspring in the future.

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No. 44: MONITORING STEROID HOROMONES IN EYES TO EVALUATE REPRODUCTIVE STATUS.

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Monitoring reproductive status is quite important for conservation and/or population control of wild animals and evaluating natural wild animal resources for fisheries. However it is difficult to obtain blood samples to measure hormones for assessing reproductive status of wild animals. Although, many wild animals are killed by accident, disease and hunting opportunities for assessing reproductive status have been missed. Eyeballs contain vitreous humor filtrated from retinal blood, which may contain hormones comparable to serum levels. The purpose of this research was to investigate possibility of vitreous humor in evaluating reproductive status by measuring hormones. In the first experiment, we measured testosterone and progesterone levels in eyeballs and sera of male and female golden hamsters at various stages of reproduction. There was a significant correlation between eyeballs and sera in hormoral levels. In the second experiment, we investigated hormonal levels in vitreous humor and sera of sika deer (*Cervus nippon*), sacrificed for

The optimal time and site for artificial insemination in the domestic cat

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Objective of the work. This study aimed to compare pregnancy rates with fresh semen between two insemination times in relation to induction of ovulation and between vaginal insemination and transcervical insemination with frozen-thawed semen in the domestic cat. The study was designed for AI in practice, when the average total number of spermatozoa varies between ejaculates and males.

Materials and methods. Generalised anaesthesia was induced in female domestic cats with xylazine and ketamine. Intravaginal insemination (IVI) using fresh semen was performed on the day the 100 IU hCG was administered (n=7) and 28 hrs after the hCG administration (n=8). Semen was collected from four males with electroejaculation. A total of 6.8 to 22 million (13.5±5.4) fresh spermatozoa per ejaculate were vaginally inseminated. An open-ended tomcat catheter (Sovereign, Kendall, Mansfield, USA) was used for IVI by inserting the catheter toward the cervix until no further cranial movement could be achieved. Semen from another male was collected and frozen with $20x10^6$ spermatozoa/straw. IVI (n=12) and transcervical insemination (IUI) (n=12) with frozen-thawed semen was performed 28 hrs after hCG administration. A transcervical catheter was comprised of a polyethylene dog urinary catheter (1.5-mm diameter) and a closed-ended tomcat catheter (Buster, Kruuse, Denmark) (modified from ref.1). Semen was frozen-thawed according to Axnér et al (2). Blood samples were collected on the day the insemination was done and 30-40 days after insemination. Serum was analysed for oestradiol-17β and progesterone concentration using luminescence immunoassay. A progesterone concentration of above 40 nmol/L on day 30-40 after insemination indicated the occurrence of ovulation. Ultrasonography was used to identify a pregnancy on day 30-40 after insemination.

Results. Injection with hCG induced ovulation in 69.2% of the females (27/39). The ovulation rates and pregnancy rates after anaesthesia and IVI with fresh semen at the time of hCG injection or 28 hours were similar. The frozen semen had a motility of 80-90% after thawing. IUI with frozen-thawed semen of 20 million spermatozoa provided a pregnancy rate of 50% while IVI with frozen-thawed semen did not result in any pregnancies.

Table 1. Pregnancy rate of cats inseminated

Artificial insemination	No.of cats administered hCG	No. of cats ovulated	Type of semen used	Pregnancy rate
IVI and hCG	7	5	Fresh	40% (2/5)
hCG and IVI 28 hrs later	8	6	Fresh	50% (3/6)
IVI	12	8	Frozen	0% (0/8)
IUI	12	8	Frozen	50% (4/8)

Conclusion. IVI using fresh semen can be performed either on the same day of ovulation induction using hCG or 28 hrs after hCG administration. Pregnancy results after AI with frozen-thawed cat semen confirms the preserved fertility of spermatozoa frozen with the protocol used. When frozen-thawed semen (20 million spermatozoa) is used, the semen should be deposited in the uterus.

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Effects of recombinant human follicle stimulating hormone on developmental competence of cat oocytes

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In the past two decade, *in vitro* culture system for domestic cat oocytes has been dramatically improved, although overall success of this technique has been variable primarily depending on a number of factors such as oocyte quality and culture condition. The aim of this study was to examine the effect of recombinant human follicle stimulating hormone (rhFSH) on developmental competence of cat oocytes in terms of *in vitro* maturation and fertilisation rates. In experiment I, cumulus oocyte complexes (COCs) were cultured in a defined maturation medium containing with various concentrations of rhFSH (0 IU, n=103; 0.01 IU, n=82; 0.05 IU, n= 72; 0.1 IU, n=68 and 1.0 IU, n=70). After 24 h of culture, the oocytes were stained with DAPI and examined for maturation stage. In experiment II, a total of 223 COCs were first matured *in vitro* as previously described in exp. I, and then fertilised with frozen-thawed semen from a proven-fertility cat. After 24-36h post-fertilisation, presumptive zygotes were fixed and examined for fertilisation rates by means of oocyte activation, pronucleus formation and cleavage rate.

rhFSH significantly increased the overall numbers of oocytes reaching metaphase II (MII) stage and fertilisation rates when compared with controls (MII rates: 67.9% vs 38.2%; fertilisation rates: 56.7% vs 24.6%, $p<0.05$). With regard to rhFSH concentration, rhFSH at 0.05, 0.1 and 1 IU/ml yielded similar MII and fertilisation rates (~71% and 59%, respectively), while 0.01 IU/ml rhFSH demonstrated less effectiveness. In conclusion, rhFSH is capable of enhancing meiotic competence and fertilisation of cat oocytes. Developmental competence up to blastocyst stage, however, has yet to be investigated.

Keywords: follicle stimulating hormone (FSH), oocyte maturation, fertilisation, cat

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