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Polyunsaturated fatty acids influence offspring sex ratio in cows

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ABSTRACT

Dietary polyunsaturated fatty acids (PUFAs) can influence fertility in farm animals. Some evidence in mice and sheep have suggested that PUFAs may influence offspring sex ratio, which may have significant value for cattle production. To test this hypothesis, three groups of Holstein cows were supplemented with either 0%, 3% or 5% protected fat (PF) in the form of calcium salt of fatty acids (rich in omega-6) from 14–21 days pre-partum until conception. Proven-fertile frozen semen from the same ejaculate was used for insemination. Calf sex recorded at birth was 8/19 (42.1%) male offspring in the control group, increasing to 14/20 (70%, $P > 0.05$) and 17/20 (85%, $P < 0.05$) in 3% and 5% PF, respectively. To test if this effect was caused by a direct influence on the oocyte, we supplemented bovine cumulus oocyte complexes during *in vitro* maturation with either omega-3 alpha-linolenic acid (ALA), omega-6 linoleic acid (LA) or trans-10, cis-12 conjugated linoleic acid (CLA). Sex ratio of the produced transferable embryos was determined using PCR of SRY gene. Similar to the *in vivo* results, sex ratio was skewed to the male side in the embryos derived from LA- and CLA-treated oocytes (79% and 71%) compared to control and ALA-treated oocytes (44% and 54%, respectively). These results indicate that both dietary and *in vitro* supplementation of omega-6 PUFAs can skew the sex ratio towards the male side in cattle. Further experiments are required to confirm this effect on a larger scale and to study the mechanisms of action that might be involved.

1. Introduction

Various mechanisms of sex determination are present in amniote vertebrates, including genotypic, environmental (for example temperature in Crocodilian reptiles), or a mixture [1]. There is evidence that mammals also have the ability to skew their sex ratios in response to environmental conditions, a system which is thought to confer evolutionary benefits [2]. This would have the least cost to the mother if the sex ratio was adjusted close to the time of conception. Two main research lines have attempted to explain possible mechanisms involved [3]. The first is based on the finding that more dominant mothers produce more male offspring, possibly mediated via alterations in their testosterone levels [4]. The second has investigated associations between the body condition or diet of the mother and offspring sex. Within the latter category, alterations in sex ratio have been reported in response to pre-conceptual maternal diet [5–8], general dietary supplementation [9], changes in body condition [10] and diabetes [11]. More specifically, the dietary content of unsaturated fats [12], polyunsaturated fatty acids (PUFAs) [13,14], glucose [15] or fructose [16] have also been implicated. Results have not, however, always been consistent. For example, female mice supplemented with an n-6 rich diet gave birth to more female pups than male pups ($P < 0.001$) compared to control or n-3 fed mothers [13]. Others have shown a skew to the male side when a high fat diet was used in mice [5] or a high n-6 PUFA diet was fed to ewes [14]. In the latter study 69% males were recorded in the n-6 PUFA group fed as a protected soybean meal vs. 38% in the control.

Sex ratio is a key factor in cattle breeding. In the dairy industry a predominance of females is preferred as these are required for milk production. Whereas, beef cattle producers may use sexed semen to produce crossbred female replacements [17]. This has led to the development of semen sexing technologies which can reliably produce a 90% gender bias, but the sorting mechanism remains costly and pregnancy rates are significantly less than those using conventional semen

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[17,18]. Other potential methods of altering the sex ratio are therefore of significant interest.

Nutritional management can be a natural, effective and less expensive substitute for hormonal and medical intervention that can be a potential health hazard in milk and meat. Dietary PUFAs are already used to increase the energy density of the diet in cattle and also have some specific effects that are energy-independent. These include prostaglandin biosynthesis, steroidogenesis and transcriptional regulation, as well as regulation of genes involved in maternal immune response and tissue remodeling [19]. The predominant PUFA in most seed lipids is linoleic acid (LA; 18:2n-6), while α-linolenic acid (ALA;18:3n-3) predominates in most forage lipids and flaxseed. These two essential fatty acids can be metabolized in animal tissue to other forms of PUFAs. Dietary PUFA supplements in ruminants have to be protected against rumen bio-hydrogenation [20]. This process also results in synthesis of conjugated LA (CLA) from LA [21].

With respect to reproductive performance in cattle [reviewed by 22–25], some PUFA supplements were reported to have positive effects on postpartum uterine involution [26], growth rate and diameter of the ovulatory follicle [27] and *in vitro* oocyte developmental capacity [28]. Staples et al. [22] reviewed different studies examining the effect of dietary fat supplements on reproductive performance of lactating dairy cows, and reported that eleven out of twenty studies showed an average of 17% improvement in conception or pregnancy rates.

Despite the widespread use of various feeds which alter dietary PUFA concentrations and ratios, the effects of PUFAs on the sex of the offspring in cattle have not to our knowledge been investigated to date. Therefore, the aim of the present study was twofold. Firstly we examined the effect of dietary supplementation of cows with two levels of protected fat (Calcium salt of fatty acids; Magnapac) on the sex of the calves produced. Secondly we tested supplementation with either ALA, LA or CLA during bovine *in vitro* oocyte maturation media. This was to determine whether any effects on the sex ratio of the embryos produced were directly on the oocyte and to identify differential effects of different PUFAs.

2. Materials and methods

2.1. Experimental animals and feeding

The experimental work of this study was carried out at a private dairy farm in cooperation with the Animal Production Department, Faculty of Agriculture, Mansoura University. Holstein cows in late lactation, 490–540 kg LBW, 1–4 parities were individually fed according to the nutrient requirements recommended by NRC [29]. The control ration consisted of concentrate feed mixture (CFM; 19% Crude Protein), corn silage (ranged from 18–20 kg/day) and berseem hay (2 kg/day). The concentrate feed mixture (CFM) was composed of 46% yellow corn, 10% wheat bran, 10% cottonseed meal, 20% soybean, 10% horse bean, 1% NaCl, 0.1% Toxfree™ (Alfa Chemical, Mansourah, Egypt), 0.4% Premix, 1.3% sodium bicarbonate, 0.2% vitamins mixture, and 1% limestone. Roughage (silage and hay) was offered ad libitum while the CFM was offered individually for each animal twice daily before milking. This resulted in a roughage: concentrate ratio of nearly 60:40%. All animals had free access to clean drinking water and mineralized salt stone.

2.2. Experimental design

Sixty Holstein cows were stratified and randomly divided into three groups according to BW and parity. Experimental cows in group 1 (n = 20) were fed with the control diet without any fat supplementation. Groups 2 and 3 were fed with the CFM of the control diet supplemented with 3% dry matter (DM) or 5% DM respectively of a protected fat (PF) (Magnapac, Norel & Nature Comp., Madrid, Spain, a calcium salt of fatty acids). The fatty acid profile of Magnapac according to the product specifications is myristic acid (C14) 1.5%, palmitic acid (C16) 44.0%, stearic acid (C18) 5.0%, oleic acid (C18:1) 40.0% and linoleic acid (C18:2) 9.5%. The net energy for lactation (NEL) was 1.76, 1.82, and 1.84 Mcal/kg DM in control, 3% PF and 5% PF diets, respectively. The experimental feeding period started at 14–21 days pre-partum and continued up to 120 day-post-partum or conception (1–3 inseminations). The nutrient requirements were adjusted every two weeks according to changes in milk yield. Cows were inseminated by artificial insemination after detection of estrus according to the traditional am–pm rule. The same proven-fertile frozen semen source was used in all treatment groups. Cows that did not conceive at first AI were re-inseminated at the next estrous. Cows that did not return to estrus after insemination were examined transrectally by ultrasonography to confirm pregnancy at 35 days post insemination. A minimum of one and a maximum of three inseminations per conception were required. During the post-partum period, live body weight (LBW) was measured and the body condition score was estimated on a scale of 1–5 (1 = emaciated, 5 = extremely fat) by the same trained technician at 15, 30, 45 and 60 days in milk (DIM). One cow in the control group was excluded from the study due to repeat breeding. The sex of the offspring was then recorded at birth.

2.3. Chemicals and reagents used for the in vitro experiment

All chemicals and reagents were purchased from Sigma Chemical Company (Poole, Dorset, UK) unless otherwise stated.

2.4. Collection of oocytes

Bovine ovaries were collected immediately after slaughter from a local abattoir and transported within 2 h to the laboratory in phosphate buffered saline (PBS) at 37 °C. Ovaries were washed in PBS and 70% ethanol. COCs were aspirated from antral follicles (3–8 mm in diameter) and only grade-I COCs were used for the experiment [30]. Serum-free TCM-199 medium supplemented with 20 mM HEPES and 0.4% (w/v) BSA was used during COC selection and washing.

2.5. In vitro maturation and PUFA supplementation

Selected COCs were cultured in four-well dishes (NUNC, Thermo Fisher Scientific, Loughborough, Leicestershire, UK) in serum-free maturation medium (TCM-199) supplemented with 10 μg/mL LH (Leutropin; Bioniche Animal Health, Belleville ON), 10 μg/mL FSH (Follitropin; Bioniche), 1 μg/mL oestradiol, 0.6% (w/v) fatty acid-free BSA, and 50 μg/mL gentamycin. Stock solutions of alpha-linolenic acid (ALA; n-3 18:3), linoleic acid (n-6; 18:2) and trans-10, cis-12 conjugated linoleic acid (CLA; n-6 18:2) were prepared in DMSO (100 mM). They were added to maturation media at a final concentration of 50 μM. Media was incubated without COCs for 2 h at 38.5 °C to allow binding of PUFAs to BSA which acts as a carrier. DMSO was added to the control group at the same concentration used with PUFAs (0.05%). We have previously shown that DMSO at this concentration has no effect on maturation and embryo development compared to DMSO-free controls [31]. COCs were incubated in maturation media containing DMSO or PUFAs for 24 h at 38.5 °C under 5% CO2 in humidified air. A total of 773 COCs were used in this experiment.

2.6. In vitro fertilization and embryo culture

In vitro matured oocytes (in the presence or absence of 50 μM ALA, LA or CLA) were fertilized with frozen semen from a single bull as previously described by Fouladi-Nashta and Campbell [32]. Briefly, swim up technique was used to select motile spermatozoa using calcium-free medium (for 45 min). Supernatant was then centrifuged at 300 × g at room temperature and spermatozoa were re-suspended (at a concentration of 1×10^6 sperm/mL) in fertilization medium (Fert-

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