Effect of copper and thiomolybdates on bovine theca cell differentiation *in vitro*

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Abstract

Subfertility that will respond to appropriate copper supplementation is a widespread problem in the national dairy herd. The aims of this study were to determine the effect of copper and/or copper chelating thiomolybdates on LH-induced differentiation by looking at the effects on androgen production, steroidogenic enzymes (cytochrome P450 17 α -hydroxylase and cytochrome P450 side-chain cleavage) and lysyl oxidase mRNA expression in cultured theca cells maintained under serum-free conditions.

The effect of thiomolybdates and copper on LH differentiation was investigated by supplementing (ammonium) tetrathiomolybdate to optimum theca cell culture media at 0–100 μ g/ml, copper (chloride) at equimolar concentrations (0–51.6 μ g/ml) or equimolar combinations of both media. Lysyl oxidase mRNA expression was investigated with semi-quantitative RT-PCR, whilst expression of cytochrome P450 17 α -hydroxylase and cytochrome P450 side-chain cleavage mRNA was examined using real time PCR. Both PCRs used bovine specific primers and cell lysates obtained from bovine theca cells cultured for 6 days and in the presence or absence of the 100 μ g/ml dose of thiomolybdate and equimolar copper thiomolybdate.

Thiomolybdate depressed androstenedione production in a dose-dependent manner at doses greater than 1 μ g/ml

at 96 h ($P < 0.05$); doses above 20 μ g/ml were all greatly reduced at all time points and at 192 h when related to numbers of cells ($P < 0.001$). Copper alone had no effect at physiological doses, but the use of the equimolar copper thiomolybdate reversed the effect of tetrathiomolybdates on androstenedione production at the 20 μ g/ml dose. Thiomolybdate supplementation, with and without copper, had no significant effect on the level of lysyl oxidase or cytochrome P450 side-chain cleavage expression. However, cytochrome P450 17 α -hydroxylase expression was significantly increased ($P < 0.05$) by tetrathiomolybdate, possibly due to a local regulatory system.

In conclusion, these results demonstrate that thiomolybdates can prevent LH-induced differentiation of bovine theca cells *in vitro* and that these effects can be ameliorated by copper supplementation. Our results also indicate that it is unlikely that the effects of thiomolybdate are mediated at the transcriptional level and further work is required to determine if thiomolybdate exerts its effects through post-translation processing or some other unrelated mechanism. Overall, these data support the hypothesis that copper responsive subfertility results from perturbation of the normal pattern of ovarian steroidogenesis.

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Introduction

The declining fertility of the national dairy herd is a major problem in the United Kingdom (Royal *et al.* 2000). Although subfertility is a multifactorial problem, a considerable proportion of animals have been shown to respond to appropriate copper supplementation (Black & French 2000, Kendall *et al.* 2001). Under field conditions, most copper responsive conditions (conditions which strictly are not primarily a copper deficiency but are alleviated by appropriate copper supplementation) are

actually induced by molybdenum, which reacts with sulphur in the rumen to form thiomolybdates, which in turn bind copper with high affinity (Dick *et al.* 1975).

Molybdenum-induced copper depletion has been reported to reduce fertility with associated reduced conception rates, anovulation, anoestrus, decreased basal luteinising hormone (LH) levels, reduced peak surge of LH and a reduction in the number and frequency of LH pulses (Phillippo *et al.* 1987). Du Plessis *et al.* (1999a) have also shown that behavioural anoestrus occurs relatively quickly, within 6 weeks, in sheep fed a high molybdenum

and sulphur diet, with true anoestrus occurring after a much longer supplementation time in the trial. Du Plessis *et al.* (1999b) also showed that ovaries were reduced in size and have a decreased response to a follicle-stimulating hormone (FSH)-induced superovulation regimen in the molybdenum and sulphur supplemented groups. Whilst molybdenum-induced copper depletion may also have central effects via the hypothalamus–pituitary axis on LH secretion, many of the above ‘symptoms’ are consistent with reduced ovarian oestradiol secretion. The absence of oestrus in animals with copper responsive subfertility could be attributed either to attenuation of the pre-ovulatory oestradiol surge that induces oestrous behaviour and the LH surge or to perturbation of the mechanisms within the central nervous system that result in the behavioural response to this endocrine stimulus (Alexander *et al.* 1980). Moffor and Rodway (1991) investigated the effect of intravenous administration of tetrathiomolybdate to lambs but found no effect of this treatment on either pulsatile LH or surge release of LH in response to gonadotrophin-releasing hormone or oestradiol challenge. These data suggest that copper responsive subfertility is unlikely to act centrally. Williams (2004) has shown that feeding differing amounts of copper, sulphur, iron and molybdenum alters the amount of copper and molybdenum within the ovary. Increasing the amount of molybdenum fed significantly increased the molybdenum and copper content of the ovary. Although only molybdenum and not thiomolybdate was actually measured, it is likely that a large proportion of the molybdenum is in the form of thiomolybdates (Williams 2004).

We have previously reported (Kendall *et al.* 2003) that thiomolybdates depressed the FSH-induced differentiation of bovine granulosa cells cultured in a serum-free system for 192 h. This was observed as a reduction in oestradiol production and an alteration in cellular morphology with the loss of the characteristic clumping seen in granulosa cells cultured under serum-free conditions (Gutierrez *et al.* 1997a, 1997b). An ameliorating effect of copper on thiomolybdate doses was also reported, as was expression of lysyl oxidase mRNA occurring in granulosa cells, both freshly isolated and throughout 192 h of culture.

Differential expression of the copper-containing enzyme lysyl oxidase was reported by Slee *et al.* (2001) whilst investigating the genes associated with gonadotrophin-induced ovarian somatic cell differentiation in rodents. Lysyl oxidase is essential for the stabilisation of the extracellular matrix (ECM) as it is the enzyme responsible for the cross linking of collagen and elastin monomers. Its action is via the oxidative deamination of peptidyl lysyl groups and the activity has been shown to be linked to increased copper intake (Rucker *et al.* 1998). Thiomolybdates have also previously been shown to affect lysyl oxidase and subsequent collagen and elastin formation, often observed as symptoms of clinical copper deficiency

which include cardiac problems, bone weakness and joint abnormalities (Underwood 1981). The ECM is also considered as an active regulator of cell migration, division, differentiation, death and anchorage with significant and dramatic changes in the remodelling of the extracellular matrix occurring during ovarian follicle development (Rodgers *et al.* 1998). Collagen type IV, laminin and fibronectin are thought to be present in lamina-like material in the theca matrix and this basal lamina seems to be constantly remodelled as the follicle matures (Rodgers *et al.* 1999).

Although we have previously shown the effect of thiomolybdates on granulosa cells (Kendall *et al.* 2003), it is also possible that thiomolybdates have effects on other steroidogenic somatic cells. The theca cells are responsible for the secretion of androgens, which are in turn used as the substrate for oestradiol production by granulosa cells (Baird & McNeilly 1981). Theca cells secrete androstenedione as their main steroidal product (McNatty *et al.* 1984). Therefore, a depression in androgen production may become a limiting factor in the secretion of oestrogens and may lead to a depression in ovarian oestrogen production contributing to the symptoms of copper responsive subfertility.

Our hypothesis is that the effects of thiomolybdate-induced copper deficiency on fertility are mediated by perturbation of gonadotrophin-induced steroid production by the somatic cells of the preovulatory follicle. In the present study, we aim to determine whether copper and thiomolybdate supplementation effects androgen production, steroidogenic enzyme and lysyl oxidase mRNA expression by cultured theca cells maintained under serum-free conditions.

Materials and Methods

Effects of thiomolybdates and copper on cultured theca cells

Unless otherwise stated all reagents were purchased from Sigma-Aldrich. Bovine ovaries were obtained from a commercial abattoir. Follicles between 2 and 5 mm in diameter were dissected from a number of ovaries to negate any individual animal effects, and theca cells were isolated using the ovine tissue procedure of Campbell *et al.* (1998) adapted for use with bovine tissue. Briefly, the dissected follicles were hemisected in Dulbecco's phosphate-buffered saline without calcium and magnesium (dPBS[−]), the granulosa cells were removed by repeated flushing through the barrel of a 1 ml syringe and the theca shells were dispersed by incubation with 10 ml of an enzyme cocktail consisting of 5 mg/ml collagenase II, 1 mg/ml hyaluronidase, 1 mg/ml protease, 0.2 mg/ml deoxyribonuclease I, and 10 µl/ml fetal calf serum (FCS) (Invitrogen) in dPBS[−] (Campbell *et al.* 1998). Dispersion was halted by the addition of 2 ml FCS and the cells were washed twice in culture media before resuspension and

counting using a trypan blue viability stain. Theca cell viability is normally greater than 95% and cells were plated at a density of 75 000 viable cells/well in a 96-well flat bottom plate (Nunc; Nunc, Roskilde, Denmark) with 250 µl theca culture medium (DMEM:Hams F12 with sodium bicarbonate and 15 mM HEPES+1mg/ml BSA, Penstrep (100 U/ml penicillin, 0.1 mg/ml streptomycin), 0.365 mg/ml L-glutamine, 5 µg/ml transferrin (Calbiochem, Nottingham, Notts, UK), 0.1 µg/ml selenium) with optimum doses of 10 ng/ml insulin, 1 ng/ml LR3-insulin-like growth factor-I (LR3-IGF-I) (Bachem, St Helens, Merseyside, UK) and 0.1 ng/ml ovine LH (NIDDK-oLH-S26, bioactivity 2.3 IU/mg) to induce theca cell differentiation (Campbell *et al.* 1998). To four wells per replicate plate (4 plates), treatments were applied as follows: ammonium tetrathiomolybdate ((NH₄)₂MoS₄) (TTM) was added to the media at concentrations of 0, 0.1, 1, 5, 20 and 100 µg/ml (termed TTM-0, TTM-0.1, TTM-1, TTM-5, TTM-20, TTM-100), tissue culture grade copper chloride hydrate (CuCl₂) (Cu) was added at equimolar equivalents with concentrations of 0, 0.0516, 0.516, 2.08, 10.32 and 51.6 µg/ml (termed Cu-0, Cu-0.1, Cu-1, Cu-5, Cu-20, Cu-100) and equimolar concentrations of the two compounds (CuTTM) mixed to form CuTTM-0, CuTTM-0.1, CuTTM-1, CuTTM-5, CuTTM-20, CuTTM-100 treatments. The cells were cultured for 144 h with 175 µl (70%) media changed every 48 h for fresh treatment media. Removed media were retained, and stored at -20 °C for subsequent analysis of androstenedione concentrations. Cell numbers were estimated using the neutral red cell viability stain at the end of the culture (144 h) (Campbell *et al.* 1996).

Androstenedione assay

Androstenedione concentrations were assayed using a previously validated double antibody radioimmunoassay (Campbell *et al.* 1990). The sensitivity of the assay was 39 pg/ml and the intra- and interassay coefficients of variation were both less than 15%. Androstenedione concentrations were expressed as total production for all time points with concentrations at the 144 h time point also being expressed on a 'cell number' basis. All androstenedione concentrations were naturally logged to obtain a normal distribution prior to statistical analysis

Expression of mRNA for lysyl oxidase, cytochrome P450 side-chain cleavage and cytochrome P450 17 α -hydroxylase in theca cells

Theca cells were isolated as described above and cells were plated at a density of 5×10^5 viable cells/well in a 24-well flat bottom plate (Nunc) with 1 ml theca culture medium with optimum insulin, LR3-IGF-I and oLH of 10, 1, and 0.1 ng/ml respectively to induce theca cell differentiation (Campbell *et al.* 1998). Media (800 µl, 80%

of well volume) were changed every 48 h where appropriate. The effect of thiomolybdate on lysyl oxidase, cytochrome P450 17 α -hydroxylase and cytochrome P450 side-chain cleavage expression of theca cells was determined by the addition of ammonium tetrathiomolybdate ((NH₄)₂MoS₄) (Sigma-Aldrich) at concentrations of 100 µg/ml (TTM-100) to the media, equimolar concentrations of copper were added for a copper thiomolybdate treatment (CuTTM-100) and no supplement was added to give a no treatment control (NT). These treatments were applied to three replicate cultures. At each time point (16, 24, 48, 96, 144 and 192 h), after removal of culture media (retained and stored at -20 °C for subsequent androstenedione assay), cells from each well were harvested into 150 µl RTL lysis buffer (RNeasy; Qiagen) containing 1% β -mercaptoethanol and retained at -20 °C for subsequent analysis. Total RNA was isolated from the lysed cell samples using the RNeasy mini kit (Qiagen) and protocol. First strand cDNA synthesis was performed using iT 1st strand synthesis kit (ABgene, Epsom, Surrey, UK). As analysis is semi-quantitative, random hexamers (Promega) were used to prime the cDNA strand as an 18S internal standard (QuantumRNA 18S rRNA Universal Internal Standard; Ambion, Huntingdon, UK) was used in all cases. All PCR amplifications were performed using bovine-specific cDNA distinguishing primers.

Semi-quantitative lysyl oxidase mRNA expression analysis was performed by conventional PCR and utilised a primer set targeting a region spanning the 3rd (sense, 5'-TTCCTACCAAGTCGACCAAGA-3') to the 6th exon (antisense, 5'-TGCATAAGCGTGATGTCCTG-3') and deriving a 399 base pair amplicon product that was beyond optimal real-time size parameters. Both the cytochrome P450 17 α -hydroxylase and cytochrome P450 side-chain cleavage expression analyses utilised real-time PCR technology and were performed on a Quanta detection system (TECHNE; Duxford, Cambridgeshire, UK) with QuantiTect SYBR Green (Qiagen) fluorescent label. The cytochrome P450 17 α -hydroxylase reactions utilised the sense primer 5'-CAAGGATGGCAACCTGAAG-3' and the antisense primer 5'-CAGAGAGAGAGGCTCGGACA-3' which amplified a 115 base pair product, while the cytochrome P450 side-chain cleavage reaction used the sense primer 5'-TTCGCCACATCGA GAACTT-3' and the antisense primer 5'-CCTCGAA CTTGAAGAGATGG-3' and derived a 117 base pair amplicon. In order to measure reaction efficiency, a standard curve was generated for each gene using standards of 10⁴, 10³, 10², 10¹, and 1 pg cDNA. Each reaction was run in duplicate and the mean log cDNA concentrations versus cycle threshold were plotted. The PCR products were electrophoresed on 2% agarose gels containing ethidium bromide, and visualised under UV light. Subsequent analysis was by laser densitometry. PCR products from parallel gels were excised and purified using QIAquick Gel extraction kit (Qiagen). Product identities

Figure 1 The mean ($\pm$ S.E.M.) numbers of theca cells cultured under optimum serum-free conditions at the end of culture at 144 h with Cu added to the media (shaded bars), CuTTM added to the media (open bars) and TTM added to the media (solid bars). The relative doses are equimolar concentrations equivalent to tetrathiomolybdate in $\mu\text{g/ml}$. No data points are significantly different ($P > 0.05$).

generated by each primer set were subsequently confirmed by sequencing.

Statistical analysis

Statistical analysis was carried out with SPSS (Chicago, IL, USA) using a univariate general linear model (GLM) for single time point analysis and repeated measures GLM for multi-time point analysis. The analysis was blocked by individual culture plate. Individual group comparisons were carried out using the Bonferroni method.

Results

Cellular morphology and cell number

The theca cells all assumed the characteristic dispersed monolayer of flattened fibroblastic cells observed in this serum-free system (Campbell *et al.* 1998). There were no apparent alterations in cellular morphology between the treatment groups. Cell numbers are shown in Fig. 1 and show no significant effect of any treatment or dose on the numbers of cells present after 144 h of culture.

Androstenedione production

Theca cells from the small antral follicles cultured serum-free in the presence of optimal doses of LH, insulin and

IGF-I, shown previously to induce cellular differentiation (Campbell *et al.* 1998), displayed the expected induction of androstenedione production with increasing time in culture, with maximal levels after 144 h (Fig. 2). The 0.1 and 1 doses of TTM had little effect on this induction profile, whilst the TTM-5 dose showed a significant reduction ($P < 0.01$) at 96 h compared with the 0, 0.1 and 1 doses, and at 144 h compared with the TTM-1 dose ($P < 0.01$). Androstenedione was further significantly reduced at the TTM-20 dose ($P < 0.001$) and was virtually undetectable at the TTM-100 dose ($P < 0.001$) for all three time points compared with the 0, 0.1 and 1 doses.

Copper alone had no significant effect on androstenedione production at all time points at any dose apart from the Cu-100 dose ($P < 0.001$) and the Cu-20 dose which was higher than the Cu-0.1 dose at 96 h ($P < 0.05$) (Fig. 2b). Similarly, when related to cell number at 144 h (Fig. 3) only the Cu-100 dose had any effect on androstenedione production ($P < 0.001$).

The effect of combining Cu and TTM on androstenedione production is shown in Fig. 2c. These results show no significant differences in androstenedione production for the 0, 0.1, 1, 5 and 20 doses, apart from the CuTTM-20 having increased androstenedione production over the CuTTM-0 dose at 96 h ($P < 0.05$). However, the CuTTM-100 dose significantly depressed androstenedione production ($P < 0.001$) at all time points without (Fig. 2c) and with (Fig. 3) correction for cell number.

Figure 2 The mean ($\pm$ S.E.M.) androstenedione production (pg/ml) of the theca cells cultured under optimum serum-free conditions with (a) TTM added to the media, (b) Cu added to the media, and (c) CuTTM added to the media for 48 (shaded bars), 96 (open bars) and 144 (solid bars) hours. The relative doses are equimolar concentrations equivalent to tetrathiomolybdate in $\mu\text{g/ml}$. Within the same time (but across all three treatments), data points not sharing a common letter are significantly different ($P < 0.05$).

Figure 3 The mean ($\pm$ S.E.M.) androstenedione production on a cell number basis (pg/1000 cells) after 144 h of serum-free culture under optimum conditions with Cu added to the media (shaded bars), CuTTM added to the media (open bars) and TTM added to the media (solid bars). The relative doses are equimolar concentrations equivalent to tetrathiomolybdate in $\mu\text{g/ml}$. Data points not sharing a common letter are significantly different ($P < 0.05$).

In comparison with TTM alone, the CuTTM treatment prevented the depression observed at the 20 dose ($P < 0.001$) across all three time points (Fig. 2a,c). A similar effect was observed when data were expressed in relation to cell number (Fig. 3). Further the TTM-5 dose differed from the CuTTM-5 dose at the 96 h ($P < 0.001$) and 144 h ($P < 0.05$) time points.

Correction of these data for cell number after 144 h of culture (Fig. 3) had little effect on the overall treatment response, with no significant difference for the 0, 0.1 and 1 doses whereas doses of TTM greater than 20 resulted in a marked inhibition in androstenedione production ($P < 0.001$).

Lysyl oxidase expression

Lysyl oxidase (399 bp amplicon) and the 18S internal positive control (317 bp amplicon) were expressed across all the time points (0, 16, 24, 48, 96 and 144 h) of the culture in non-treated controls (data not shown). The effects of thiomolybdate supplementation or thiomolybdate with equimolar copper supplementation (both at 100 dose) on lysyl oxidase expression at 96 h are shown in Fig. 4. It can be seen that expression of lysyl oxidase mRNA for the TTM-100 dose was not significantly different from either the non-treated or the CuTTM-100-treated cells.

Cytochrome P450 17 α -hydroxylase and cytochrome P450 side-chain cleavage expression

The relative mRNA expression of the steroidogenic enzymes cytochrome 17 α -hydroxylase and cytochrome

P450 side-chain cleavage at 96 h are shown in Fig. 5. Both TTM and CuTTM had a slight depressive effect on cytochrome P450 side-chain cleavage ($P = 0.22$). Conversely, TTM supplementation significantly ($P < 0.05$) up-regulated the cytochrome P450 17 α -hydroxylase expression whereas the CuTTM had no effect.

Figure 4 (a) The gel product of the PCR showing lysyl oxidase (LOX; 399 bp) and 18S internal standard (317 bp) amplicons. M, molecular weight marker lane. (b) The mean ($\pm$ S.E.M.) relative expression of LOX to 18S internal standard from theca cells cultured for 96 h with optimum media (NT), the addition of 100 $\mu\text{g/ml}$ ammonium tetrathiomolybdate (TTM) and the addition of 100 $\mu\text{g/ml}$ ammonium tetrathiomolybdate and equimolar copper chloride (CuTTM). LAU, light arbitrary units.

Figure 5 The relative expression of cytochrome P450 side-chain cleavage (solid bars) and cytochrome P450 17 α -hydroxylase (shaded bars) after 96 h of serum-free culture, with no treatment (NT), tetrathiomolybdate at 100 μ g/ml dose (TTM) or tetrathiomolybdate at 100 μ g/ml dose with equimolar copper (CuTTM). Different letters indicate a significant difference ($P<0.05$) for cytochrome P450 17 α -hydroxylase. There was no significant difference between cytochrome P450 side-chain cleavage results. LAU, light arbitrary units.

Discussion

The results within the present experiment show that TTM is able to prevent LH-induced differentiation of bovine theca cells in culture and that copper can ameliorate the effect of TTM on androstenedione production. These depressive effects could not be attributed to changes in mRNA expression for the steroidogenic enzymes cytochrome P450 17 α -hydroxylase and cytochrome P450 side-chain cleavage, nor the copper-dependent enzyme lysyl oxidase.

The effects of thiomolybdates in the whole animal have previously been shown to include reduced conception rates, anovulation and anoestrus, which were associated with a decreased release of LH (Phillippo *et al.* 1987). The pre-ovulatory oestradiol peak is the endocrine signal responsible for induction of both behavioural oestrus and the preovulatory LH surge (Baird & McNeilly 1981) and the dominant follicle in ruminants is the source of over 96% of ovarian oestradiol secretion (Baird & Scaramuzzi 1976). Theca cells do not produce oestradiol directly, but produce androgens that are used as a substrate by granulosa cells in the production of oestradiol (Baird & McNeilly 1981). It has been estimated that androstenedione represents 82% of the steroid secreted by the theca cells, testosterone accounting for only 5% (McNatty *et al.* 1984). Therefore, a reduction in the production of androstenedione, as we have reported for TTM doses greater than 5, is likely to limit the production of oestradiol due to substrate limitation. The results of the present study are consistent with an effect of

molybdenum-induced copper deficiency on the development of the preovulatory follicle, especially steroid secretion, and would therefore be expected to perturb both behavioural oestrus and ovulation to a varying degree, as has been observed in animals with molybdenum-induced copper responsive subfertility (Phillippo *et al.* 1987, Du Plessis *et al.* 1999a, 1999b). Further, it is well established that ovarian oestradiol plays a key role in controlling gamete transport and in priming the uterus for implantation, so that even if ovulation does occur, fertility rates are likely to be impaired if preovulatory oestradiol production is sub-optimal (Hafez 1980). Again, this is one of the key symptoms of copper-responsive subfertility (Du Plessis *et al.* 1999a) and further studies are required to examine the effects of molybdenum-induced copper deficiency on the pattern of development and steroidogenic capacity of ovulatory follicles *in vivo*.

Examination of the effects of TTM treatment on expression of two key steroidogenic enzymes that result in androstenedione biosynthesis, cytochrome P450 17 α -hydroxylase and cytochrome P450 side-chain cleavage seems to preclude the level of mRNA expression as an explanation of the depressive effects of TTM treatment.

Copper may be acting indirectly either through mitochondrial energy (cytochrome c oxidase requires three copper ions) or the cofactors to cytochrome P450 side-chain cleavage, which are ferredoxin and ferredoxin reductase (copper is extensively involved in iron metabolism).

However, cytochrome P450 side-chain cleavage, which is often regarded as the rate limiting step in this steroidogenic pathway (Miller 2002), was only marginally depressed ($P=0.22$) by exposure to TTM, whereas, conversely, cytochrome P450 17 α -hydroxylase expression was markedly up-regulated ($P<0.05$). This unexpected finding raises the interesting possibility that cytochrome P450 17 α -hydroxylase expression is under the control of a local regulatory feedback system that has been perturbed by treatment with TTM. Thus, from our results this perturbation is likely to act via low concentrations of the enzyme product i.e. androstenedione. More direct experimental analysis is required to investigate this interesting and novel observation in more detail. Further work is also required to determine whether TTM treatment acts to depress LH-stimulated thecal cell androgen production by affecting the enzymatic activity of cytochrome P450 17 α -hydroxylase and cytochrome P450 side-chain cleavage, rather than their level of mRNA transcription.

The results of this study also show that mRNA for the copper-dependent enzyme lysyl oxidase is expressed by freshly isolated and cultured theca cells. This is not surprising as dramatic changes and remodelling of the extracellular matrix occur during ovarian follicle development (Rodgers *et al.* 1998). The ECM is also considered as an active regulator of cell migration, division, differentiation, death and anchorage. Collagen type IV, laminin

and fibronectin have previously been shown to be secreted by cultured granulosa cells (Rodgers *et al.* 1995, 1996) and are also thought to be present in lamina-like material in the theca matrix (Rodgers *et al.* 1999). The basal lamina seems to be constantly remodelled as the follicle matures (Rodgers *et al.* 1999) which may explain a putative role of lysyl oxidase in androstenedione production.

However, in this paper, although we have shown that cultured theca cells express lysyl oxidase and that thiomolybdates depress the steroid production of the cells, thiomolybdates did not affect lysyl oxidase expression to a significant extent. Therefore, these results indicate that it is unlikely that these effects of TTM are mediated by lysyl oxidase at the transcriptional level and further work is required to determine if TTM exerts its effects through lysyl oxidase translation, post-translational processing or some other unrelated mechanism. Harlow *et al.* (2003) showed that increased androgen (5 α -dihydrotestosterone) increased the level of mRNA expression for lysyl oxidase in rat granulosa cells in a serum-supplemented culture. In the present study, the increased androgen production found for the non-treated cells in comparison with the tetrathiomolybdate (TTM-100)-treated cells was found to have no effect on lysyl oxidase mRNA expression. However, the differences between this work and that of Harlow *et al.* (2003) may be explained by the use of different cell types (granulosa vs theca), different species (rat vs bovine) and different culture types (serum containing vs serum free). Slee *et al.* (2001) found no lysyl oxidase mRNA expression in rat theca cells cultured in a serum containing culture, whereas they did find lysyl oxidase expression in rat granulosa cells. The contamination of the serum-free theca cell culture type used in this study with granulosa cells is negligible (approx. 1%) and would not explain the lysyl oxidase expression found.

In a previous study we have similarly reported that TTM can inhibit FSH-induced oestradiol production by cultured granulosa cells, and that these effects can be ameliorated by co-incubation with copper (Kendall *et al.* 2003). The fact that TTM has similar effects in both granulosa and theca cells suggests that copper responsive subfertility may be mediated through effects on both these cell types, but their relative importance remains unclear. However, an interesting difference between the cell types observed in these studies was an apparent increase in sensitivity to copper for theca cells. In theca cells in the present paper a copper dose of 100 (51.6 μ g/ml) was used as the highest dose and had the effect of reducing androstenedione production without affecting the number of cells. However, in granulosa cells (Kendall *et al.* 2003) a similar copper dose had no apparent effect on cell number, oestradiol production or morphology of the cells and a much higher copper dose of 1000 (516 μ g/ml) was required to elicit these responses. This effect may possibly be due to the inhibition by copper of cytochrome P450

catalysed reactions. Kim *et al.* (2002) found a 30 μ M dose of CuCl₂ completely depressed cytochrome P450 activity in an *in vitro* assay. As the granulosa cell culture has testosterone substrate in the media it therefore relies only on cytochrome P450 aromatase activity, whereas the theca cell culture relies on *de novo* synthesis of steroids and therefore many cytochrome P450 catalysts.

An argument often used in cell culture studies is that effects are observed at non-physiological, often pharmacological doses. In the granulosa cell studies (Kendall *et al.* 2003) we calculated the expected physiological doses working from published concentrations of copper and molybdenum in blood and came to the conclusion that the physiological range for thiomolybdate and copper were equivalent to our doses of TTM-0.14 to TTM-8.1 and Cu-0.31 to Cu-0.77 respectively. This means that the differential effects seen in this paper of a small but significant decrease at a dose of TTM-5, with a highly significant decrease for the TTM-20 dose are at the higher end of the calculated physiological dose and can therefore be expected to cause similar effects *in vivo*.

In conclusion, the results within the present experiment show that TTM is able to prevent LH-induced differentiation of bovine theca cells in culture and that copper can ameliorate the effect of TTM on androstenedione production. These depressive effects did not appear to be mediated by any fall in mRNA expression for the steroidogenic enzymes cytochrome P450 17 α -hydroxylase and cytochrome P450 side-chain cleavage. Further, the experiments demonstrated that although the copper-dependent enzyme, lysyl oxidase, is expressed by theca cells, TTM does not exert its effect by modulating the level of lysyl oxidase mRNA expression. Overall, these data support the hypothesis that the effect of TTM on fertility may be mediated via perturbation of the normal pattern of ovarian steroidogenesis.

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