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Effects of testosterone either alone or with IGF-I on growth of cells derived from the proliferation zone of regenerating antlers *in vitro*

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Summary Deer antlers are male secondary sexual *characters* and are the fastest growing mammalian tissue. As such, both androgens and growth factors play a major role in antler development. The timing of the antler cycle is controlled by the seasonal fluctuations of testosterone, and the actual growth of antlers is mainly stimulated by growth factors including insulin-like growth factor-1 (IGF-I). However, whether or not testosterone at low levels plays a growth-promoting role during antler formation is controversial. In the present study, we took an *in vitro* approach to investigate whether testosterone either alone or with IGF-I had mitogenic effects on mesenchymal or cartilaginous cells derived from the proliferation zone of regenerating antlers. In addition, a binding assay was carried out to determine whether the specific binding sites for testosterone were preserved after cell disaggregation. The results showed that testosterone either in physiological concentrations or at low levels did not exert direct mitogenic effects on antler cells derived from the proliferation zone in serum-free medium *in vitro* ($P > 0.05$), even if the specific binding sites for testosterone in these cells were well preserved. Likewise, testosterone in a very wide range of concentrations not only failed to enhance ($P > 0.05$), but at certain levels (0.1–5 nM) impaired the mitogenic effects of IGF-I on these antler cells *in vitro* ($P < 0.001$). Therefore, these results support neither a conclusion that low level testosterone has growth-promoting effects on antler formation nor the hypothesis that testosterone effects may be achieved through sensitizing these antler cells to the mitogenic effects of IGF-I.

Key words: deer antler, IGF-I, testosterone, *in vitro*, mitogenesis.

INTRODUCTION

Deer antlers are bony organs, which are cast and regenerate each year. Unlike horns which grow from the base, the antler growth centre is located in its tip.[1] Banks and Newbrey[2] classified the antler tip into four zones, namely the zones of proliferation, maturation, hypertrophy and calcification. The proliferative zone consists of three layers. These are, distoproximally, reserve mesenchyme, precartilage and cartilage. Antler growth is mainly achieved through the activity of the cells residing in the proliferation zone, particularly in the layer of reserve mesenchyme.

With the exception of reindeer, antlers are only grown by males. It has been convincingly demonstrated that the timing of the antler growth cycle is strictly under the control of androgen hormones.[3] However, whether androgens play a trophic role in antler growth has been a subject of intensive debate. As early as 1943, Wislocki thought that there must be a

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non-gonadal factor involved in antler growth, which was named antler growth stimulus (AGS). This is because antler growth always takes place at a time when deer reproductive organs are most inactive and castration does not seem to affect further antler growth. However, Bubenik[4] claimed, based on his findings from the testosterone measurement of three castrated white-tailed deer, that AGS was in fact testosterone. He hypothesized that testosterone had dual roles in antlerogenesis: at a low level it was growth promoting, but at a high level it resulted in antler calcification. Further, he suggested that the reason castrated deer could continue to grow antlers was that there was a sufficient quantity of androgens produced by the deer adrenal glands.

Suttie *et al*[5] reported that the seasonal peak of insulin-like growth factor-I (IGF-I), a non-gonadal factor, almost perfectly coincided with the peak of antler growth rate. As IGF-I plays a growth-promoting role in cartilage formation and the antler growth centre is mainly composed of cartilage, they suggested that IGF-I might be the AGS. IGF-I receptors were subsequently located in the growing antler tissues.[6] More convincing results to support the notion that IGF-I might be the AGS were subsequently reported by Sadighi *et al*.[7] They found that IGF-I increased the proliferation of the mesenchymal and cartilaginous cells derived from antler proliferation zone in a dose-dependent manner. Taking these IGF-I results together with their extensive study on the role of steroids in antler growth, Suttie *et al*[3] concluded that antler growth, at least in red deer, does not appear to require testosterone. If a trophic role were to be considered, testosterone would exert a priming effect on the cells in the proliferation zone, which would then become more responsive to IGF-I during the antler growth phase. This conclusion was further supported by the study reported by Li *et al*, [8] where testosterone alone in serum-free medium did not show any mitogenic effects on the mesenchymal cells from pedicles or first antlers. However, the physiological concentration of testosterone (2.88 ng/ml) used in their study was 7.2 times higher than the low level ($\leq$0.4 ng/ml) suggested by Bubenik.[4]

In order to determine whether low levels of androgens are required for antler growth, Bartos *et al*[9] compared the subsequent antler growth of castrated fallow bucks with or without further treatment with synthetic antiandrogen cyproterone acetate (CA). The results showed that the CA treatment not only significantly lowered testosterone levels but substantially reduced antler growth, when compared with untreated castrates. Therefore, they concluded that a plasma androgen concentration above a minimal threshold value seems to be a necessary prerequisite for antler growth at a normal rate. However, it is not known whether the growth-promoting role played by this minimal threshold testosterone is through a direct or indirect pathway.

The aim of this study was to use *in vitro* techniques to culture mesenchymal and cartilaginous cells from the proliferation zone of regenerating antlers, to investigate (1) whether testosterone alone, including physiological and low levels, could stimulate thymidine incorporation into the antler cells in serum-free medium, (2) whether low level testosterone could play a role in sensitizing the antler cells to the mitogenic effects of IGF-I and (3) whether different levels of IGF-I could affect possible mitogenic effects of testosterone on the antler cells.

MATERIALS AND METHODS

Antler tissue was obtained from farmed red deer stags 60 days after casting of the previous hard antlers. Primary cell cultures were prepared as previously described by Sadighi *et al*.[7] Briefly, mesenchymal and cartilaginous layers were sampled by dissection and washed with a balanced salt solution. Thereafter, the tissues were chopped and incubated in a 25 cm^2 flask (Falcon, Lincoln Park, NJ, USA) containing 10 ml digesting medium (45% BGJb, 45% F_{12} nutrient, 10% foetal bovine serum (FBS), 100 u/ml penicillin, 100 μg/ml streptomycin and 200 u/ml collagenase (all these reagents from Sigma, St Louis, MO, USA). The flasks were incubated at 37°C for 24 h and then centrifuged to remove the supernatant. The disaggregated cells were then resuspended in the culture medium (digesting medium without collagenase).

The cells were seeded in culture flasks at a density of 2×10^4 cells/cm^2 in the culture medium. The flasks were incubated in a humidified incubator in 95% air and 5% CO_2 at 37°C. The culture medium was changed every 3 days. Cells were subcultured on reaching confluence using trypsin–EDTA (Sigma). Aliquots of cells were frozen in liquid nitrogen in the freezing medium (culture medium plus 10% dimethyl sulphoxide (Sigma)). In the present study all cells had been through two passages. Cell viability was measured using trypan blue and was always above 85%.

Three experiments were set up to evaluate the mitogenic effects of testosterone either alone or with IGF-I on the antler cells from the proliferation zone of regenerating antlers. One experiment was set up to determine whether the specific binding sites for testosterone were preserved after the cell disaggregation. Testosterone was dissolved in 100% ethanol. For each experiment, mesenchymal or cartilaginous cells were seeded in 24-well plates at a density of 2×10^4 cells/ml per well. Each treatment was performed in triplicate wells.

The cells were incubated for 48 h, followed by 24 h in serum-free medium (SFM: normal culture medium with 0.1% bovine serum albumin (BSA) instead of 10% FBS) before proceeding with the different treatments. In the first three experiments, 2 h before the termination of the incubation, [^{3}H]thymidine (85 Ci/mmol, Amersham) at 2.5 μCi/ml was added to each well. At the end of treatments, the cells were dissolved in 0.1 M NaOH (BDH), and the solution was counted in a β counter. Protein content was measured using the Lowry method.[10] The proliferation rate is expressed as incorporation of [^{3}H]thymidine (dpm/μg protein).

Experiment 1

The aim was to determine whether the physiological concentration of testosterone either alone or with 10 nM IGF-I could influence the proliferation of the mesenchymal or cartilaginous cells *in vitro* in a similar manner to mesenchymal cells from pedicles or first antlers.[8]

After 24 h incubation in SFM, the cells had a final incubation for 24 h in SFM containing (1) no additives, (2) 0.1% ethanol, (3) 10 nM IGF-I (a gift from Kabi Pharmacia, Sweden), (4) 10 nM testosterone (Sigma, St Louis, MO, USA), (5) 10 nM IGF-I + 1 nM testosterone or (*6*) 10 nM IGF-I + 10 nM testosterone.

Experiment 2

The aim was to determine whether any mitogenic effect on antler cells could be detected from a wide range of concentrations of testosterone alone, or a very wide range of concentrations of testosterone with 10 nM IGF-I.

Following incubation in SFM, the cells had a final incubation for 24 h in SFM containing (1) no additives, (2) 0.1% ethanol, (3) 10 nM IGF-I, (4)–(*6*) 0.1 nM, 1 nM or 10 nM testosterone, (*7*)–(*19*) 10 nM IGF-I + 0.001 nM, 0.005 nM, 0.01 nM, 0.05 nM, 0.1 nM, 0.5 nM, 1 nM, 5 nM, 10 nM, 50 nM, 100 nM, 500 nM or 1000 nM testosterone.

Experiment 3

The aim was to determine whether different levels of IGF-I could affect any possible mitogenic effect of testosterone on the antler cells.

After 24 h incubation in SFM, the cells had a final 24 h incubation in SFM containing (1) no additives, (2) 0.1% ethanol, (3) 10 nM IGF-I, (4) 0.3 nM IGF-I + 10 nM testosterone, (5) 1 nM IGF-I + 10 nM testosterone, (*6*) 3 nM IGF-I + 10 nM testosterone, (*7*) 10 nM IGF-I + 10 nM testosterone, (8) 0.3 nM IGF-I + 1 nM testosterone, (*9*) 1 nM IGF-I + 1 nM testosterone, (10) 3 nM IGF-I + 1 nM testosterone, (11) 10 nM IGF-I + 1 nM testosterone.

Experiment 4

The aim was to determine whether testosterone-specific binding sites were present in primary cultured antler cells.

Specific binding of [^{3}H]testosterone (Amersham, Auckland, New Zealand) to the mesenchymal or cartilaginous cells was investigated as follows. After 24 h incubation in SFM and immediately before the binding study, the cells were washed three times with binding buffer (containing 0.1% BSA, 0.2% azide). They were then incubated at 0°C for 1 h in binding buffer containing 1 nM [^{3}H]testosterone with or without 0.2 μM cold testosterone. Then, the cells were washed three times with PBS and dissolved in 200 μl 1 N NaOH and 100 μl was counted in a β counter.

Statistical analysis

For all experiments $\log_{10}$(dpm) was analysed by analysis of variance, with treatment structure according to the design of the experiment. These usually consisted of terms for cell line, treatment and their interaction. In addition, orthogonal contrasts among treatments were fitted to assess the linearity of dose responses and differences between other treatment groupings of interest.

RESULTS

Experiment 1

Compared with the SFM treatment, neither 10 nM testosterone nor 0.1% ethanol had a significant effect on [^{3}H]thymidine uptake for both mesenchymal and cartilaginous cells (Fig. 1). Significantly higher mitogenesis was observed for 10 nM IGF-I for both cell types ($P < 0.001$). Testosterone, at both 1 nM and 10 nM, decreased ($P < 0.001$) the mitogenic effect of 10 nM IGF-I. This inhibitory effect of testosterone on IGF-I was greater ($P < 0.01$) in mesenchymal cells than in cartilaginous cells (Fig. 1).

Experiment 2

Neither testosterone at any of the doses used nor 0.1% ethanol had a significant effect on [^{3}H]thymidine uptake in either mesenchymal or cartilaginous cells compared with SFM treatment (Fig. 2). For mesenchymal cells, doses of testosterone between 0.1 nM and 5 nM significantly decreased the mitogenic effect of 10 nM IGF-I ($P < 0.001$), while the lower and higher doses of

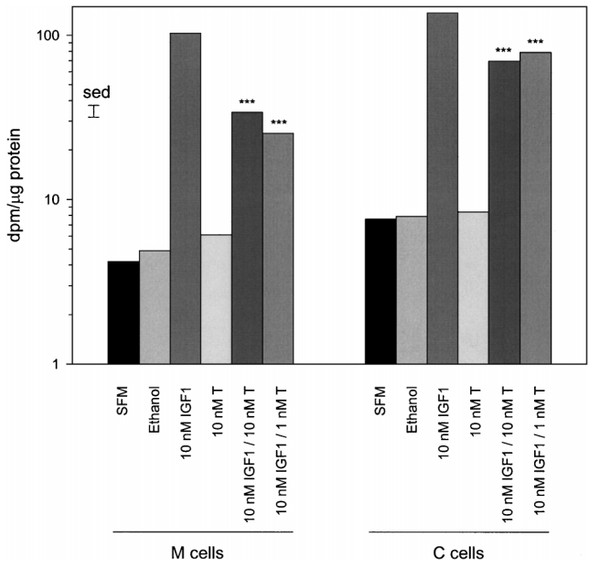

Fig. 1 Mitogenic effects of physiological concentrations (1 and 10 nM) of testosterine (T) alone or with 10 nM IGF-I on mesenchymal (M) or cartilaginous (C) cells ($n = 3$). sed, standard error of difference; ⋆⋆⋆, $P < 0.001$ compared with 10 nM IGF-I.

testosterone did not modify the mitogenic effects of 10 nM IGF-I ($P > 0.05$). For cartilaginous cells, doses of testosterone between 0.005 nM and 5 nM significantly decreased the mitogenic effect of 10 nM IGF-I ($P < 0.001$), while the lower and higher doses of testosterone did not significantly modify the mitogenic effect of 10 nM IGF-I.

Experiment 3

For both cell types, [^{3}H]thymidine uptake was significantly ($P < 0.001$) higher for 10 nM IGF-I than for the average of the treatments that included both testosterone and IGF-I (Fig. 3). Among these treatments, 1 nM testosterone had lower ($P < 0.001$) [^{3}H]thymidine uptake than 10 nM testosterone, and mesenchymal cells had lower ($P < 0.01$) [^{3}H]thymidine uptake than cartilaginous cells. For mesenchymal cells with 10 nM testosterone, a significant linear decline in response with log(IGF-I) concentration was observed ($P < 0.01$), in contrast to a significant linear increase ($P < 0.001$) for cartilaginous cells. For the 1 nM testosterone

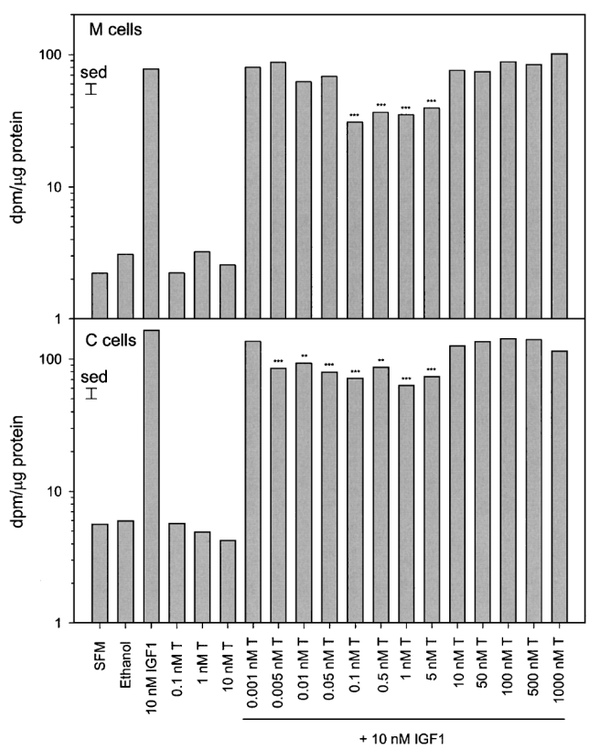

Fig. 2 Mitogenic effects of different doses (0.001–1000 nM) of testosterone (T) with 10 nM IGF-I on mesenchymal (M) cartilaginous (C) cells ($n = 3$). sed, standard error of difference; ⋆⋆, $P < 0.01$, ⋆⋆⋆, $P < 0.001$ compared with 10 nM IGF-I.

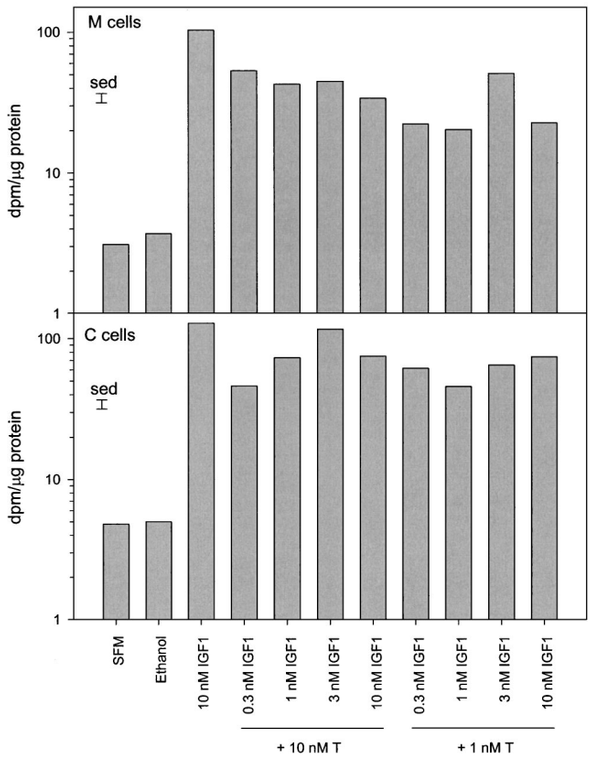

Fig. 3 Mitogenic effects of physiological concentrations (1 and 10 nM) of testosterine (T) with different doses (0.3, 1 and 10 nM) IGF-I on mesenchymal (M) or cartilaginous (C) cells ($n = 3$). sed, standard error of difference.

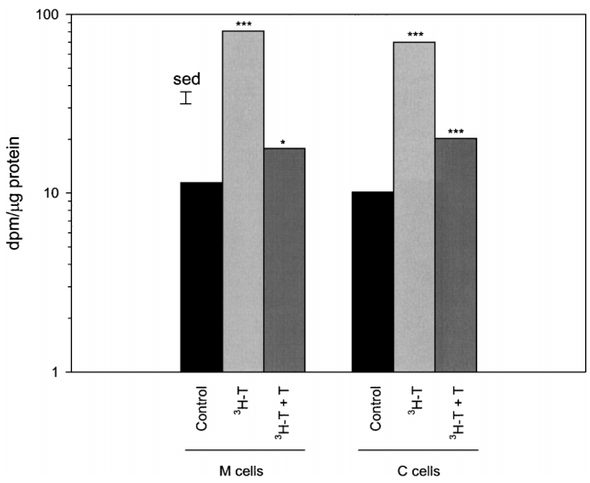

Fig. 4 Testosterone (T) specific binding to mesenchymal (M) or cartilaginous (C) cell ($n = 3$) [^{3}H]-T, tritium-labelled testosterone; sed, standard error of difference; ⋆, $P < 0.05$, ⋆⋆⋆, $P < 0.001$ compared with control.

treatment, the linear contrast in log(IGF-I) concentration was not significant for either cell type.

Experiment 4

Specific binding of [^{3}H]testosterone to mesenchymal or cartilaginous cells was demonstrated (Fig. 4). In both mesenchymal and cartilaginous cells, [^{3}H]testosterone was displaced by the excess of unlabelled testosterone ($P < 0.01$).

DISCUSSION

Deer antlers are male secondary sexual characters and the fastest growing mammalian tissue. As such, both androgens and growth factors play a major role in antler development. It is agreed that the timing of the antler cycle is controlled by the seasonal fluctuations of testosterone, and the actual growth of antler is mainly stimulated by IGF-I. However, whether or not testosterone at a low level plays a growth-promoting role during antler formation is still controversial.[9]

The present study clearly demonstrated that testosterone, in a wide range of concentrations (0.1 nM (0.0288 ng/ml)–10 nM (2.88 ng/ml)) *in vitro* in SFM, did not exert direct mitogenic effects on either mesenchymal or cartilaginous cells derived from regenerating antler tips. In addition, in our previous studies we found that the physiological concentration of testosterone (2.88 ng/ml) does not have mitogenic effects on the same type of cells from the subsequent regenerating[11] or from the first generated antlers.[8] Based on these results we conclude that testosterone, either at a physiological concentration or at a low level as suggested by Bubenik[4] (≤0.4 ng/ml) and by Bartos *et al*[9] (0.01–0.2 ng/ml), does not exert direct mitogenic effects *in vitro* on antler cells derived from the proliferation zone. However, one may argue that antler cells do not react directly to testosterone, because a change in structure and function of these primary cultured antler cells has taken place after the removal from their extracellular matrix. One possible change is the loss of specific androgen binding sites if the *in vitro* selection favors androgen-independent cells, as shown in some cases of primary cultured prostatic epithelial cells.[12] However, experiment 4 showed the specific binding sites of testosterone are well preserved in the present study. Therefore, it is unlikely that the failure to demonstrate a dependence on androgens for growth *in vitro* of these antler cells is due to the loss of specific androgen binding sites.

Bartos *et al*[9] conclude in their report that a minimum threshold concentration of testosterone is a necessary prerequisite for normal antler growth to occur. Based on our *in vitro* findings, the growth-promoting effect of this minimum threshold concentration of testosterone, if it exists, would not be through direct stimulation but via an indirect pathway. Bartos *et al*[9] thought that the possible role of low concentrations of testosterone in antler growth might be to sensitize antler mesenchymal cells and chondroblasts to the mitogenic effect of IGF-I. This conclusion was based on their study which showed that although at the same level of IGF-I, the control group, which had a higher plasma testosterone level, formed bigger antlers than the CA-treated group. However, in the present study, we cannot confirm this sensitizing role of testosterone using a very wide concentration range (0.001 nM (0.000288 ng/ml)–1000 nM (288 ng/ml)) *in vitro* on antler cells (experiment 2). If a sensitizing role occurs, at least one concentration of testosterone within our tested range plus 10 nM IGF-I should have a higher proliferation rate than the 10 nM IGF-I alone treatment. However, this is not observed in our study. Even at different levels of IGF-I (0.3 nM–10 nM), the sensitizing role of testosterone is not detected (experiment 3). Instead, in this study we found that testosterone at certain levels (0.1 nM–5 nM) impaired the mitogenic effects of IGF-I on these antler cells. This inhibitory effect was more profound in mesenchymal cells than in cartilaginous cells (experiment 3). As antler growth is mainly achieved by the rapid proliferation of mesenchymal cells,[13] testosterone might be a major inhibitory systematic factor on antler growth. Therefore, our results do not support the hypothesis that testosterone has a mitogenic role in antler growth via the IGF-I pathway.

If testosterone does not have direct mitogenic effects on antler cells, and if testosterone cannot play a growth-promoting role in antler formation via the IGF-I pathway, what is the possible explanation of the results reported by Bartos *et al.*? In their study, deer from the control group which had significantly higher plasma testosterone level formed bigger antlers compared with those from the CA-treated group. One possibility would be the involvement of extracellular matrix (ECM) in this growth-promoting effect of testosterone, because ECM does not exist in our *in vitro* system, although it is an integral component of antler tissue *in vivo*. Firstly, ECM may mediate the testosterone mitogenic effects on antler cells. Both *in vivo*[14] and *in vitro* organ culture[15] demonstrated that testosterone has significant stimulatory effects on bone growth. However, the mechanism underlying this mediation is not known. Maor *et al*[15] found that, although testosterone can stimulate the cells of an isolated bone organ in culture to produce IGF-I, the testosterone-induced cell proliferation is sustained regardless of local IGF-I blocking. Therefore, it is unlikely that IGF-I is involved in this ECM mediation. Secondarily, testosterone may stimulate antler cells to produce more ECM *in vivo*, and the increased ECM mass would contribute to antler growth. This may be one of the explanations for the reason why antler cells possess testosterone-specific binding sites in the present study. Bubenik *et al*[16] found that testosterone most abundantly existed in the lower zone of perichondrium of growing antlers using immunohistological methods and suggested that testosterone may play an important role in antler bone matrix synthesis. Another possibility is that the antler growth-promoting effects detected in the experiment of Bartos *et al*[9] could be exerted by androstenedione rather than testosterone because they found that the level of this steroid was also significantly higher in controls than in the CA-treated group.

As well as the possibilities of other unknown indirect pathways or species dependence, an alternative explanation is that the results may be the consequence of the dosage of CA used in the treatment. The dose of CA used in their study was 1000 mg/treatment per deer at 2 day intervals until the day of antler casting (day 22 after castration) and at weekly intervals thereafter. The dosage of CA used before antler casting is 4–12 times higher than the maximal dosage used by the previous studies.[3,4,17,18] The dosage of CA used after antler casting is also significantly higher (2–4 times) than those used in these previous studies during the early antler growth phase. It is not known whether or not a high dose of CA could have serious side effects. Indeed, Bartos *et al*[9] carefully discussed this in their paper, particularly addressing the issue of the steroid-mimicking effect of high CA doses. They found no detectable side effects using such a high CA dosage in their study. However, some side effects caused by a high dose of CA on antler growth may be beyond their ability to define. If that is the case, both the initiation of antler formation and antler growth from the CA-treated deer in their study could have been affected by unknown non-physiological reactions to the extremely high dose CA, rather than by the barely detectable testosterone levels (0.01–0.2 ng/ml) considered by Bartos *et al.*[9] A similar experiment but with different levels of CA treatments would be sufficient to clarify whether or not the dosage used in their experiment was appropriate.

In conclusion, testosterone either in physiological concentration or at low levels does not exert direct mitogenic effects on antler cells derived from the proliferation zone cultured *in vitro* in SFM, even though the specific binding sites for testosterone in these cells are well preserved. Likewise, testosterone in a very wide range of concentrations not only failed to enhance but at certain levels actually impaired the *in vitro* mitogenic effects of IGF-I on these antler cells. Therefore, these results support neither the conclusion that low level testosterone has growth-promoting effects on antler growth nor the hypothesis that the testosterone effects may be achieved by sensitizing these antler cells to the mitogenic effects of IGF-I.

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