Effect of anabolics on bovine granulosa-luteal cell primary cultures

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Abstract: Granulosa cell tumours are observed with increased frequency among calves slaughtered in Northern Italy. The use of illegal anabolics in breeding was taken into account as a cause of this pathology. An *in vitro* approach was used to detect the possible alterations of cell proliferation induced by anabolics on primary cultures of bovine granulosa-luteal cells. Cultures were treated with different concentrations of substances illegally used in cattle (17β-estradiol, clenbuterol and boldione). Cytotoxicity was determined by means of MTT test, to exclude toxic effects induced by anabolics and to determine the highest concentration to be tested. Morphological changes were evaluated by means of routine cytology, while PCNA expression was quantified in order to estimate cell proliferation. Cytotoxic effects were revealed at the highest concentrations. The only stimulating effect on cell proliferation was detected in boldione treated cultures: after 48 h treated cells, compared to controls, showed a doubled expression of PCNA. In clenbuterol and 17β-estradiol treated cells PCNA expression was similar to controls or even decreased. As the data suggest an alteration in cell proliferation, boldione could have a role in the early stage of pathogenesis of granulosa cell tumour in cattle.

Keywords: Cattle - Granulosa-luteal cells - Anabolics - PCNA

Introduction

The European Community banned the use of anabolics in Europe by means of laws 96/22/EC and 96/23/EC. In other countries (*e.g.* USA, Canada, Australia and New Zealand) natural hormones, such as testosterone, 17β-estradiol and progesterone, and synthetic hormones, like trenbolone, zeranol and melengestrol acetate, are legally used as growth promoters. Despite the regulations, in Italy, exogenous sex hormones, cortisone and β-agonists are widely and illegally used in cattle for anabolic purposes during the last two months of the fattening period. Researches carried out in our Department on veal calves regularly slaughtered in our region have revealed a high frequency of lesions of the female genital tract, related to illegal use of sex hormones, sometimes in association with corticosteroids, or clenbuterol [1,2]. The reported prevalence of granulosa cell tumours in cattle revealed at the abattoir is between 0 and 0.74% [3]. They are mainly described in adult animals and, very rarely, in veal calves [4].

However, an earlier study in our Department looking at veal calves regularly slaughtered in Piedmont (Italy) revealed a dramatically higher prevalence of granulosa cell tumours (2.54%) [5].

Several factors may have played a role in the development of granulosa cell tumours in veal calves with such a high prevalence. Among these, it is probable that specific environmental factors may be a major factor in the development of granulosa cell tumours in veal calves. In the literature, a strict correlation between hormones and cancer has been revealed [6-8]. Sex hormones and gonadotropins are thought to be important in the regulation of granulosa cell proliferation and maturation [9,10]. Hormones can stimulate cell growth, even in mutated cells, so that they are considered co-carcinogens. Stimulating mitosis, hormones increase the mutation risk, increasing the number of cell divisions [6]. Usually, the greater part of the mutations is corrected by DNA repair mechanisms and, as these require prolonged times, it is assumed that the increase of cell division speed can increase the risk of mutations transfer to daughter cells. Consequently, the hormones may behave not only as co-carcinogens, but also as true carcinogens, able to provoke an increase of the risk of mutation in their target cells and to stimu-

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late, at the same time, the replication of the mutated cells [8]. Sex steroid hormones may act as growth factors and activate signalling pathways that have been shown to have a proliferative role in many cell lines [11]. Furthermore, sex steroids seem to interfere with apoptotic control mechanisms although contradictory results have been reported. Some authors found that β-estradiol inhibited apoptosis on granulosa cells [12,13], while others showed a lack of influence of this hormone [14]. As regards androgens, in some experiments they have been shown to promote granulosa cells apoptosis [15], while other authors have affirmed that they preserved granulosa cells and follicles from undergoing programmed cell death [14]. 17β-boldenone, esters of 17β-boldenone and boldione are openly sold in Italy as anabolic preparations. 17β-aldosterone improves the growth and feed conversion of cattle and therefore might be abused to achieve more efficient meat production. Boldione is also available on the Internet for use by body builders as a product with an even greater anabolic potency than 17β-boldenone itself. Like the other androgenic steroids, 17β-boldenone is classified by the International Agency for Research on Cancer as a probable human carcinogen, with a carcinogenicity index higher than that of other androgens [16].

In recent years, several models of primary granulosa cell cultures collected from different animal species have been widely used to evaluate the effects of many substances and hormones on cell proliferation and steroidogenesis *in vitro* in cattle [17,18].

Following these considerations, it should be useful to evaluate the possible involvement of anabolics in the pathogenesis of granulosa cell tumours in veal calves. Due to the difficulties of performing an *in vivo* study, the aim of the present research was to perform a preliminary assessment of the effects of anabolic substances on granulosa-luteal cell cultures, using an *in vitro* approach. The main focus was to detect the possible alterations of cell proliferation induced by anabolic substances, being this the first step reported in carcinogenesis [8]. Morphological changes were evaluated by means of routine cytology, while PCNA expression was quantified in order to estimate cell proliferation. To exclude toxic effects induced by anabolic substances and to determine the highest concentration to be tested, cytotoxicity was determined by means of MTT test.

Materials and methods

Preparation of culture medium and reagents. All the reagents were obtained from Sigma (St. Louis, MO, USA) and the disposable plastics from Nunc (Naperville, IL, USA), unless otherwise specified. The culture medium consisted of M199 medium, modified with Earl's salt, 25 mM Hepes and 1.2 g/L sodium bicarbonate, supplemented with 1% (v/v) antibiotic-antimycotic solution, 2.5 mM L-glutamine, and 10% fetal calf serum.

Follicular cells harvesting. Ovaries were obtained from healthy heifers and veal calves slaughtered at a local slaughterhouse (Turin, Piedmont, Northern Italy). The collected ovaries were transferred to the laboratory within 1 h in sterile phosphate buffered saline (PBS) containing 1 mg/L gentamicin at 25-30°C. Granulosa cells were aseptically collected using a syringe fitted with a 18-gauge needle by aspiration of follicular fluid from small (<5 mm) follicles.

Follicles with an opaque appearance, fragmented or viscous membrana granulosa were classified as atretic, according to the criteria of Kruip and Dieleman [19], and were discarded. The collected follicular fluid was then immediately placed in sterile tubes containing culture medium. To avoid cell clumping heparin was added (50 UI/mL). The cell suspension was decanted for a short time in a water bath at 38.5°C, then aliquots of sediment were transferred in a sterile 35 mm petri dish and diluted with fresh culture medium. Oocytes were discarded using a stereomicroscope (Zeiss, Jena, Germany, magnification ×120), under sterile laminar flow conditions, in order to avoid any interference with the tests.

Aliquots of purified granulosa cells were then pooled and centrifuged at 600 g for 5 min. The pellet was treated with 0.9% prewarmed sterile ammonium chloride at 38°C for 3 min to remove red blood cells and centrifuged at 600 g for 5 min. The cells were washed twice in fresh medium, counted using a haemocytometer and viability was estimated by trypan blue (0.4%) exclusion method.

Cell culture and addition of test substances. Cells were cultured with a microdrop technique, seeded in 45 μL drops at a concentration of 4.5×10^6 cells/mL, in 8-well chamber slides, to perform morphological and immunocytochemical studies, or in 96-well tissue culture plates, for MTT test.

The cell cultures were incubated in a water-saturated atmosphere of 5% CO_2 at 38.5°C for an initial 48 h period, to achieve cell adhesion. Then, one-half of the exhausted medium was replaced. The cultures were fed with fresh culture medium containing appropriate treatments, "feeding" the culture to a final volume of 90 μL. Different concentrations (from 10^{-3} M up to 10^{-9} M) of 17β-estradiol, clenbuterol or boldione were added. The plates were returned to the incubator for further 24 or 48 h. Test substances were dissolved in ethanol, and the final concentration of ethanol never exceeded 1%. Culture medium alone was added to control wells, and culture medium containing ethanol was added to relative control wells, to evaluate the influence of ethanol on cultures. Tests were carried out in 3-5 replicate wells.

Estimation of cytotoxicity. Estimation of cytotoxicity of anabolics on cell viability was determined by means of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay, according to the protocol of Mosmann [20], on cells grown on 96-well plates, after 24 or 48 h of treatment. Absorbance at 595 nm was read on a multiwell spectrophotometer (Bio-Rad, model 450, Hercules, CA, USA), with a 655 nm reference filter.

In the MTT assay, each anabolic concentration was tested in triplicate-quintuplicate measurements per experiment, and each experiment was carried out at least three times. Percent survival was calculated as the percentage absorbance of the treated wells relative to the untreated wells ±SEM. (standard error of the mean).

Cytology and immunocytochemistry. At the end of the treatment period, medium was removed from the cultures carried out in chamber slides and cells were formalin fixed. Slides were rinsed twice in PBS and stained with haematoxylin and eosin or immunocytochemistry for PCNA, to determine the proportion of granulosa-luteal cells entering the cell cycle. Slides were incubated with a solution of 3% H_2O_2 in methanol to quench endogenous peroxidase activity for 10 min. The slides were washed

three times in PBS and submitted to immunocytochemistry for PCNA, using the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA) and a specific monoclonal antibody to PCNA (Clone PC10, 1:50 in PBS, Dako, Glostrup, Denmark) for 3 h. Controls included the omission of the primary antibody and the incubation with an irrelevant isotype matched antibody. After rinsing, slides were treated with 0.5 mg/mL diaminobenzidine tetrahydrochloride in PBS and 0.3% (w/v) H_2O_2. Slides were counterstained with haematoxylin for 10 s, followed by washing and blueing in running tap water for 10 min. Finally, the slides were ethanol-dehydrated, cleared with xylene, and mounted. The slides were examined with an Olympus BX41 (Tokio, Japan) microscope and pictures were taken with an Olympus BP12 colour digital camera.

The labelling index for PCNA was calculated by counting the percentage of positive nuclei on the total number. Image analysis was carried out by means of Image-Pro Plus software (Image-Pro Plus, Media Cybernetics, Silver Spring, MD, USA). For each treatment three random microscopic fields were counted at a magnification of ×200. For each tested substance, cultures treated with three dilutions of the anabolic were counted: the lowest (10^{-9} M), a middle one (10^{-6} M), and the highest where a toxic effect of the substance was not revealed by MTT assay (referring to the paragraph Results). Results were expressed as a percentage of the control internal to the culture.

Statistical analysis. Statistical analysis was performed by means of Statgraphics Plus software (Statistical Graphic Corp., Manugistics, Inc., Rockville, MD, USA). One-way ANOVA was used to evaluate differences between treated cultures and controls, and Fisher's tests were subsequently used to make individual comparisons within a given treatment group providing the initial one-way ANOVA indicated a significant effect of that treatment.

Unless stated otherwise, numerical values are presented as means ±SEM. Data were normalized respect corresponding controls, carried out in the same conditions of the tested cultures (same inoculum, medium, time of culture) and expressed in percentage. Data were combined from three to six individual cultures and p values below 0.05 were considered to be statistically significant.

Results

MTT test showed that boldione drastically reduced cell viability, showing an extremely toxic effect at the highest concentration tested (1 mM): a 25% reduction of cell viability was shown after 24 h of culture in the presence of the anabolic, whereas it reached 75% after 48 h. Concentrations lower than 1mM did not interfere significantly with cell viability. In cultures incubated with 1 mM clenbuterol, the MTT test showed a viability reduction of 50% 24 h post treatment and 25% 48 h after, in comparison to the control examined at the same time. As for boldione, lower concentrations did not interfere significantly with cell viability. Finally, as regards 17β-estradiol, MTT suggested that there was no significant difference between treated cell viability and controls.

Following the results obtained by cytotoxicity evaluation, the highest dilutions used to quantify PCNA expression were 10^{-3} M for 17β-estradiol, and 10^{-4} M for boldione and clenbuterol, *i.e.* the highest non-toxic concentrations for the cells.

The morphological appearance of treated cultures differed substantially from control cultures, even if a small level of apoptosis was detected even in control cultures, especially after 48 h.

Cells treated with 17β-estradiol 10^{-3} M appeared foamy (Fig. 1d and 2d). The nucleus/cytoplasm ratio was strongly reduced and, especially after 48 h, the cells presented chromatin condensation, cytoplasm enlargement, and, often, even cytolysis and karyorrhexis (Fig. 1d and 2d). At the lower concentrations, the lesions progressively reduced, and in 10^{-9} M treated cells the nucleus/cytoplasm ratio was almost normal. The foamy appearance persisted in 10^{-6} M treated cells, after 24 h, where a discrete number of pyknotic nuclei was also present and even in 10^{-9} M treated cells, after 48 h cytoplasmic vacuolization could be detected.

Cells treated with clenbuterol, in high concentrations (Fig. 1c and 2c), or boldione (Fig. 1b and 2b), even in lower concentrations but for a longer time (48 h), presented many pyknotic nuclei. In clenbuterol 10^{-4} M treated cells cytoplasmic vacuolization was conspicuous after 24 h (Fig. 1c) and the maximum presence of pyknosis was detected at the same concentration after 48 h (Fig. 2c). At 10^{-6} M the number of pyknotic nuclei was fairly higher after 48 h than after 24 h. With the lowest concentration a level of chromatin condensation different from controls was revealed only after 48 h of treatment, while after 24 h the cells were comparable to control, a part form the fact that their nuclei were elongated.

Chromatin aggregation, nuclear and cytoplasmic condensation, cell shrinkage, partition of cytoplasm and nucleus into membrane bound-vesicles and fragmentation of cells into smaller apoptotic bodies characterized cells treated with boldione 10^{-4} M, especially at 24 h (Fig. 1b and 2b). These features were also present, even if to a lesser extent, in cultures treated with any lower concentration of the anabolic. Cells had a polygonal shape, rather different from the fibroblast appearance of controls, and cytoplasm was vacuolized.

Different degrees of staining were revealed by immunocytochemistry (Fig. 3). In control cultures (Fig. 3a), the percentage of PCNA positive nuclei was 25.32% ± 7.90 (range: 10.88 - 33.46) of the total number of nuclei. Fig. 4 shows the quantification for PCNA staining. The asterisks indicate statistical difference from controls. Boldione did not interfere with PCNA expression 24 h post treatment, while it was able to increase its expression in a dose-dependent manner after 48 h.

PCNA expression in clenbuterol treated cultures seemed almost constant in time, independently from examined dose. The lowest tested dose, 24 h after the treatment, provoked a reduction of about 35% in
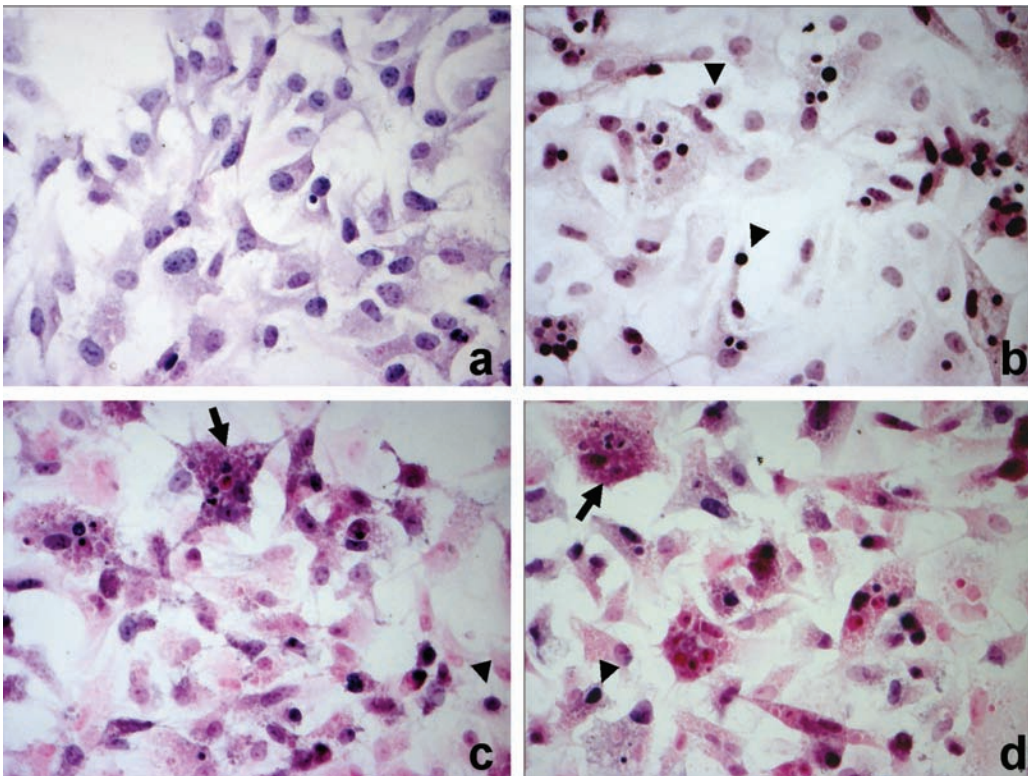
Fig. 1. Morphological appearance of cultured granulosa-luteal cells, 24 h post treatment. Haematoxylin and Eosin staining (magnification ×400). **a**. control culture; **b**. cells treated with boldione 10^{-4} M; **c**. cells treated with clenbuterol 10^{-4} M; **d**. cells treated with 17β-oestradiol 10^{-3} M. Arrow: cytoplasm enlargement, vacuolization, and karyorrhexis; arrowhead: pyknotic nuclei.
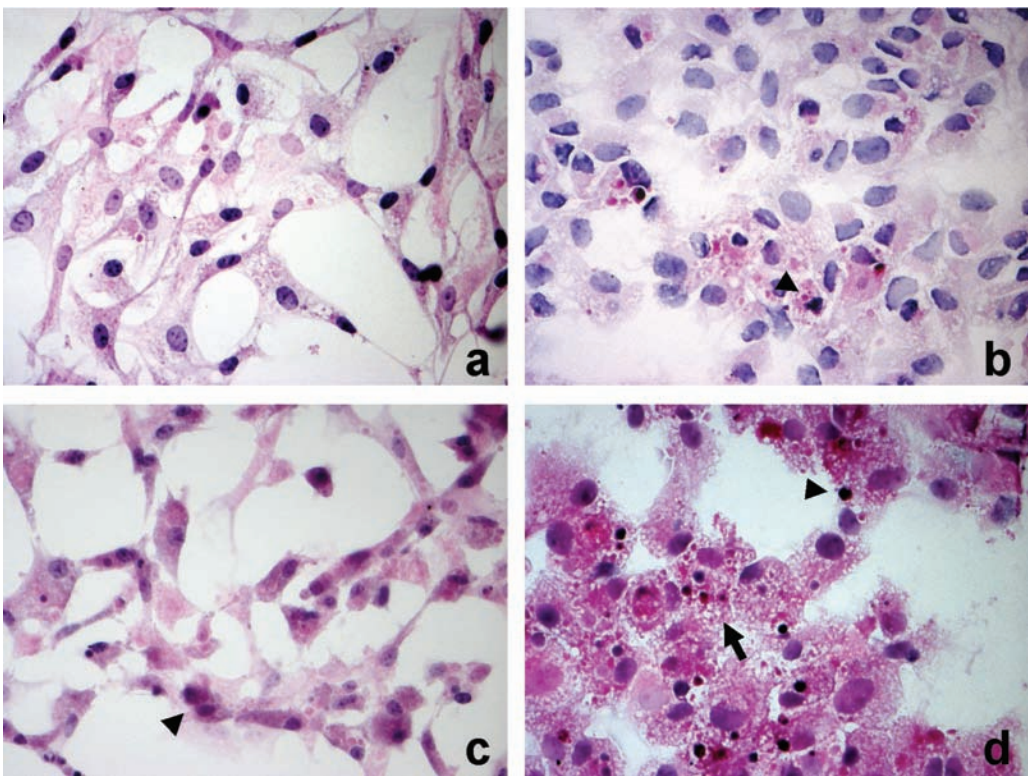

Fig. 2. Morphological appearance of cultured granulosa-luteal cells, 48 h post treatment. Haematoxylin and eosin staining (magnification ×400). **a**. control culture; **b**. cells treated with boldione 10^{-4} M; **c**. cells treated with clenbuterol 10^{-4} M; **d**. cells treated with 17β-estradiol 10^{-3} M. Arrow: cytoplasm vacuolization, foamy appearance, and karyorrhexis; arrowhead: pyknotic nuclei.
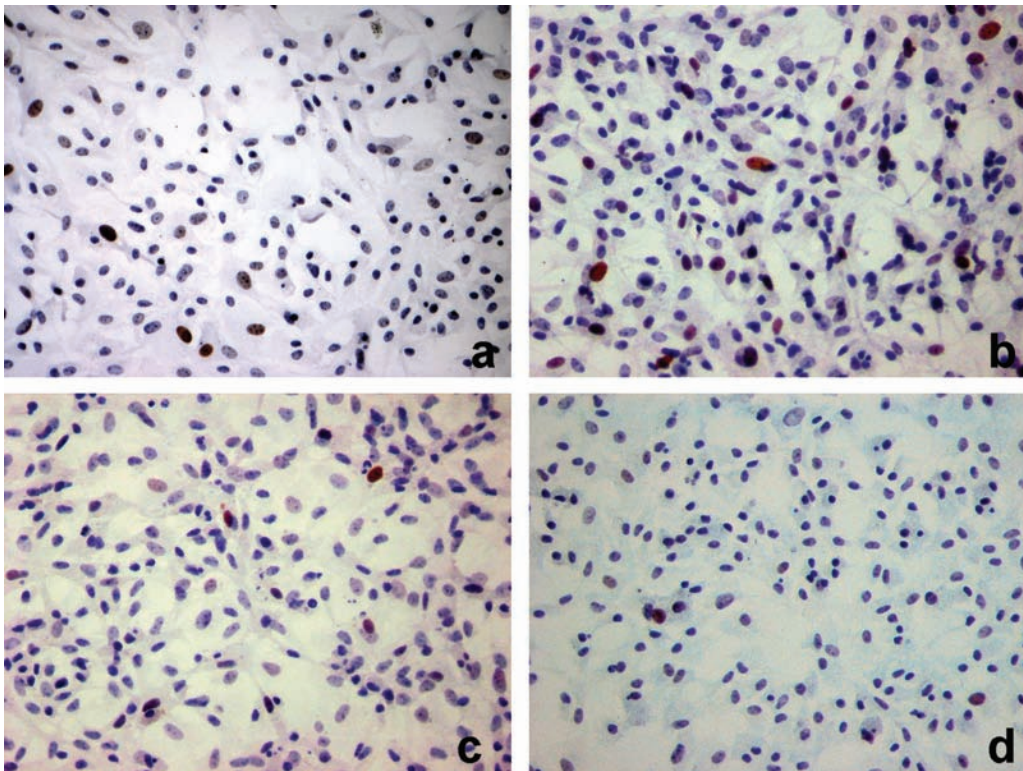
Fig. 3. Microphotographs of cultured granulosa-luteal cells. PCNA immunostaining (magnification x200). **a**. control culture, 48 h of culture; **b**. cells treated with boldione 10^{-4} M, 48 h post treatment; **c**. cells treated with clenbuterol 10^{-9} M, 24 h post treatment; **d**. cells treated with 17β-estradiol 10^{-3} M, 24 h post treatment. Brown staining of PCNA positive cells.

immunocytochemical staining. 17β-estradiol neither showed enhancement effects on PCNA expression in treated cultures; indeed, in some conditions (1 mM and 1 nM at 24 h, and 1 μM and 1 nM at 48 h) a reduction of the immunocytochemical staining was evident, reaching the 40%, compared to control.

Discussion

The aim of the present research was to evaluate the possible influence of anabolics on granulosa cells proliferation in veal calves, using an *in vitro* approach. A complete culture medium model allowed to obtain homogeneous cultures, with cells more viable even on morphological examination compared to serum free cultures. Serum deprived granulosa cells, in fact, undergo spontaneous apoptosis [21] and after only 5 h of serum deprivation, 20-30% of granulosa cells are apoptotic. In the present investigation, the experiments were conducted only on follicular fluid aspirated from small (<5 mm) follicles, which are more responsive to hormonal treatments [22], and oocytes were eliminated in order to avoid any interference with the tests. In fact it has been reported that some factors released form oocytes stimulate granulosa cell proliferation [23], and interfere with their differentiation [24,25]. Culturing cells in drops, in addition to the high seeding

cell density and to cell hyperplasia phenomena, allowed to obtain a sub-confluent status in 48 h, despite the scarce granulosa cells proliferation ability *in vitro*. Treated cultures appeared morphologically different from control cultures. Chromatin aggregation, and nuclear and cytoplasmic condensation could be detected in cultures treated with each one of the anabolics, especially at higher concentrations, suggesting a possible activation of apoptotic pathways. Cells treated with high concentrations of 17β-estradiol and clenbuterol were altered, and presented pyknotic nuclei, as cultures treated with boldione, even in low concentrations for 48 h. The foamy appearance and the strong reduction of nucleus/cytoplasm ratio presented by cells treated with high concentrations of 17β-estradiol could also be related to an increased production of hormones, being the 17β-estradiol strongly involved in granulosa-luteal cells metabolism *in vivo*. The proportion of granulosa-luteal cells entering the cell cycle was determined using the cell proliferation marker PCNA, the cofactor of delta DNA polymerase, active both in replicative DNA synthesis, during S phase, and in reparative DNA synthesis, associated to damaged DNA repair mechanisms [26]. Increased PCNA expression correlates with the earliest signs of granulosa cell growth and its immunoreactivity remains prevalent in granulosa and theca cells of follicles in
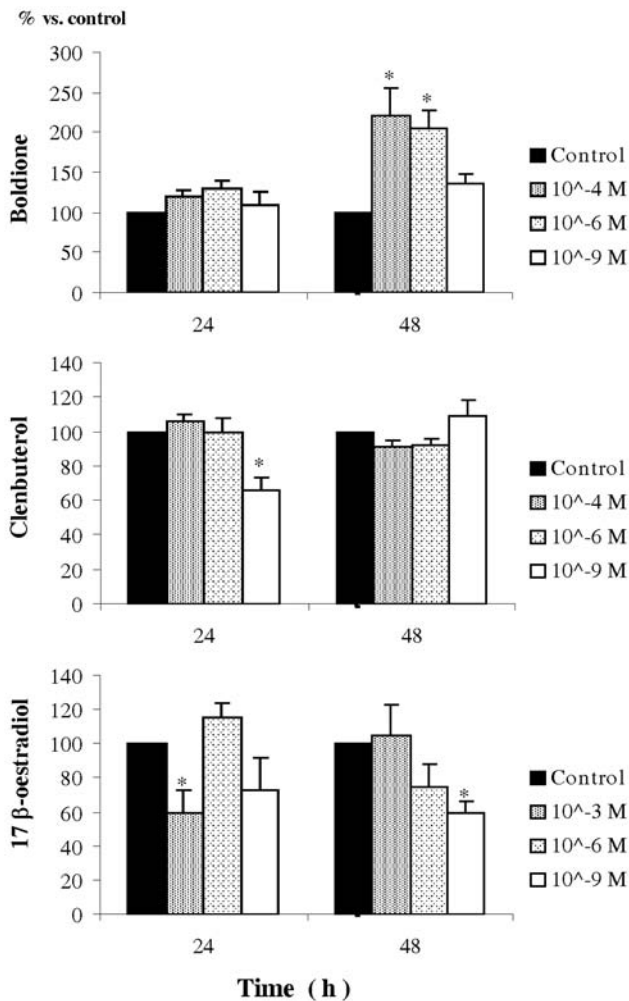
Fig. 4. Evaluation of PCNA immunostaining of cultured granu-losa-luteal cells. Values were obtained calculating the percentage referred to internal control and expressed as mean ±SEM. Asterisks indicate a statistically significant difference (p<0.01) compared to controls. Brown staining of PCNA positive cells.

subsequent stages of follicle growth, with a progressive decrease related to follicular atresia [27,28].

MTT test suggested a strong toxic effect for boldione and clenbuterol in high concentrations, so we excluded these cultures from the PCNA labelling analysis. Boldione treated cells compared to controls showed a higher degree for PCNA, after 48 h. Low doses of clenbuterol reduced PCNA expression in granulosa-luteal cells after 24 h, as 17β-estradiol, at some dilutions. Clenbuterol (10^{-3} M-10^{-8} M) and 17β-estradiol (10^{-7} M and 10^{-8} M) effects have been evaluated previously using a model represented by human tumour mammary cells [29]. In that study, as in the present one, the highest concentration of clenbuterol (10^{-3} M) caused a high cell mortality (84%), while at a 10^{-4} M dose an antiproliferative effect was evident (probably due to the toxicity of the anabolic). In addition, 10^{-7} M and 10^{-8} M concentrations significantly increased cell proliferation in a dose-related way.

As our results never showed an enhancement of PCNA expression for both clenbuterol and 17β-estra-diol, but instead an antiproliferative effect, we would exclude their role in the pathogenesis of granulosa cell tumour in cattle. The dramatic enhancement of cell proliferation due to the boldione treatment of cultures for 48 h, as revealed by PCNA expression, would suggest its role in the early stage of pathogenesis of gran-ulosa cell tumour in cattle. The simultaneous presence of apoptosis and proliferation could represent a mechanism of cell resistance to tumourigenic stimuli.

A future target could be the evaluation of other proliferation markers involved in follicular development *in vivo*, to confirm these preliminary results, and to verify the effects provoked on primary granulosa-luteal cell cultures by other illegally used anabolics (and their cocktails). Encouraging preliminary results suggest the value of continuing with the present research in order to deepen our knowledge of anabolic treatments, and better understand how to control the illegal use of anabolics and to verify their potential risk for the consumer health.

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