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Corticosterone 21-acetate *in vivo* induces acute stress in chicken thymus: cell proliferation, apoptosis and cytokine responses

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Summary. *In vivo* effects of acute stress induced by corticosterone 21-acetate in male *Gallus domesticus* thymus are studied and the steroid actions are evaluated in terms of cell proliferation, apoptosis and cytokine response in 10- and 21-day-old chickens. Steroid treatment induced thymocyte apoptosis and cell death decreased in the cortical-medullar direction and was more evident in younger animals. 24 h after treatment, the observed effect was reversed. The mitotic activity and thymic cells containing cytokine-like molecules were also affected. Indeed, the acute stress stimulated cytokine immunoreactivity to anti-IL-1α, IL-6 and TNF-α antibodies both in epithelial cells and interdigitating cells located in medullar and cortical-medullar regions. The increased cytokine expression observed after 12 h was maintained after 24 h. The comparison between 10- and 21-day-old chickens showed a lower number of cells containing cytokine-like molecules in younger specimens. The present findings suggest that cytokines activated by acute stress *in vivo* could contribute to restoring immunological homeostasis and influence thymic glucocorticoid-mediated functions.

Key words: *Gallus domesticus*, Thymus, Corticosterone 21-acetate, Apoptosis, Mitosis, Cytokines

Introduction

Glucocorticoids (GC) are powerful regulators of immune functions and important stress mediators. The effects of these molecules on immunity are rather confused and derive from *in vitro* studies on immune cells and from *in vitro* and *in vivo* actions of synthetic GC that may not mimic the role exerted by endogenous molecules *in vivo* (McEwen et al., 1997). Current data indicate that their actions on immune functions are dependent on various factors, such as hormone dose, type of steroid, target immune tissue and the interactions with other signal molecules. GC are now considered modulators of immune functions, acting both as enhancing and/or inhibiting factors, rather than immunosuppressive, as previously thought (McEwen et al., 1997).

Various studies have focused on the relationships between GC and the normal development of T-cells in the thymus. The thymic microenvironment is an excellent example of a complex network of interactive signalling systems that influence the effects of GC on thymocytes, i.e. the induction of apoptosis (Savino and Dardenne, 2000). The intrathymic production of GC (Vacchio et al., 1994), the presence of steroidogenic enzymes, and studies using GC synthesis inhibitors and receptor antagonists (Godfrey et al., 2001) indicate a role for these molecules in thymopoiesis. Nevertheless, their precise function is controversial, and conflicting data on involvement in thymocyte development have been summarized by Godfrey et al. (2001). The difficulty in discovering exactly how GC exert their effects is also complicated by the feedback regulation of systemic GC on the hypothalamus-pituitary-adrenal axis that is, in turn, activated by other immune mediators (i.e. cytokines). In addition to activating GC release, pro-inflammatory cytokines, such as tumour necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6, can also regulate GC receptor-gene expression and function in a number of cell and tissue types, including T-cells (Kam et al., 1993), suggesting multiple interactions between stress mediators, i.e. cytokines and GC (Pariante et al., 1999; Webster et al., 2001). The role of cytokines in thymic physiology is as yet only partly understood. Some studies indicate that cytokines may inhibit GC-induced lymphocyte apoptosis (Mor and Cohen, 1996), but in other reports no protection was found (Lanza et al., 1996) neither was apoptosis induced (Lenardo, 1991).

Most data concerning the role and the interactions between cytokines and GC are derived from studies using synthetic adrenal steroids that are not bound by corticosteroid-binding globulins and this allows equal access to receptors in all immune compartments

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(McEwen et al., 1997). In contrast, endogenous steroids bind corticosteroid-binding globulins that buffer organs of immune system, such as thymus, from too much of the endogenous GC producing a protective mechanism that is not active in the case of synthetic corticosteroids (McEwen et al., 1997).

In the present investigation, the *in vivo* effects of acute stress induced by corticosterone 21-acetate in chicken thymus are studied. The steroid actions are evaluated in terms of cell proliferation, apoptosis and cytokine response in animals of different age.

Materials and methods

Specimens of male *Gallus domesticus* 10 and 21 days after hatching were used. According to previous data (Franchini and Ottaviani, 1999), the two groups of animals were chosen before the thymus age-related changes. Ten 10-day-old and ten 21-day-old animals were intramuscularly injected with two different doses of corticosterone 21-acetate (Sigma, St. Louis, MO, USA): 10 and 20 mg/Kg in 50% ethanol. Two other groups of ten animals were used as controls: 10 chickens were not treated and 10 received ethanol solution alone. The thymic lobes were dissected out 12 and 24 h after treatment and were either fixed in different fixative solutions (Bouin's mixture, neutralized 10% formalin) or immediately frozen. Mayer's hematoxylin and eosin histological stain was carried out on paraffin sections, and the immunocytochemical procedure described in detail elsewhere (Ottaviani et al., 1995), was performed on both paraffin and frozen sections. The following polyclonal antibodies (pAb) were used: goat anti-interleukin (IL)-1α pAb (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:1000); goat anti-tumour necrosis factor (TNF)-α pAb (Santa Cruz Biotech.) (1:1000); goat anti-IL-6 pAb (Santa Cruz Biotech.) (1:1000); and goat anti-caspase-3 p20 pAb (Santa Cruz Biotech.) (1:1000). Primary antibodies were incubated overnight at 4 °C and the immunoreactivity was visualized by an immunoperoxidase technique using the avidin-biotin peroxidase complex (Hsu et al., 1981). Controls of immunocytochemical reactions were performed by substituting primary antibodies with non-immune sera.

The number of apoptotic and mitotic cells present in thymic sections were counted in 100 cortical microscopic fields and the differences between control and treated samples were statistically analyzed by ANOVA. The number of cells (cells/mm^2 thymic section) immunoreactive to anti-cytokine antibodies was also recorded. The areas of thymic sections were calculated using the Scion image for Windows software (Scion corporation, Maryland, USA)

Results

The histological examination of thymic sections from 21-day-old animals 12 h after corticosterone 21-acetate treatment (20 mg/Kg) revealed the presence of apoptotic cells, mostly thymocytes, in areas of the cortex from the subcapsular to the inner regions (Fig. 1A-C). These cells were rarely seen isolated, but were arranged in groups to form a multicellular complex with a pale epithelial cell and were mostly located in the outer cortex (Fig. 1A,B). A cortical-medullary gradient of apoptotic cells was observed. In the inner cortex and medulla there were fewer or isolated cells with an apoptotic phenotype (Fig. 1C, D) than in the outer cortex. The morphological observations were also supported by the immunocytochemical reaction with the anti-caspase-3 antibody (Fig. 1E). The statistical analysis of the number of apoptotic cells counted revealed significant differences between treated and control sections (Fig. 2A). No modification was detected in the thymus of ethanol-injected animals in comparison to controls (Fig. 2A). 24 h after the treatment, the observed apoptotic effect was reversed (Fig. 2A), and the structural organization of thymic lobules was comparable to controls (Fig. 1F). With regard to mitosis, the highest proliferative activity was observed in cortical thymic areas of controls. 12 h after treatment the mitotic cell number had significantly decreased and remained lower than controls after 24 h (Fig. 2B).

The same steroid dose injected in younger (10-day-old) animals provoked the same effects as those observed in 21-day-old specimens. However, in the younger animals, a higher number of areas containing apoptotic cells was seen after 12 h both in the cortex (Fig. 4A) and in the medulla (Fig. 4B), even if, after 24 h, the apoptotic cell number showed values similar to controls (Fig. 2C).

With regard to the molecules which were immunoreactive to the tested cytokines, they were present in a few single epithelial cells (Fig. 4C) and in interdigitating cells (Fig. 4D) distributed both in the medullar and cortical-medullar regions. It should be remembered that the nature of these thymic cells has been previously described (Ottaviani et al., 1997a). After corticosterone treatment of 21-day-old animals, no differences in thymic location and in immunoreactive cell types were detected, but the number of positive cells increased after 12 h and persisted after 24 h (Fig. 3A-C). In younger animals, the increase in immunoreactive cells was evident after 24 h (Fig. 5A). The comparison between 10- and 21-day-old *G. domesticus* showed a lower number of cells containing cytokine-like molecules in younger animals (Fig. 5A).

In both age groups, the lower corticosterone dosage (10 mg/Kg) showed no differences in apoptotic trends and mitotic patterns when compared to the higher concentration. A similar number of apoptotic phenotypes was observed after 12 h in the two age groups, but this was lower than with the higher dose (Fig. 5B). No differences in cytokine immunoreactive cells were observed after a 10 mg/Kg treatment of 21-day-old animals. The only significant changes were found after 12 h in 10-day-old animals (Fig. 5C, for example).

Discussion

The present study suggests that acute stress induced by corticosterone 21-acetate treatment stimulates thymocyte apoptosis in male *G. domesticus*, and that the sensitivity to the steroid decreases in the cortical-medullar direction. Cortical immature thymocytes, particularly cells bearing a double-positive phenotype, are more sensitive to GC-induced cell death than mature T-cells (Cohen et al., 1970; Cohen, 1989). Although the molecular basis of such a biological response is not clear, this behaviour has been partially explained by proposing a role for GC in the mechanisms of thymocyte development and selection that occurs in the organ (Godfrew et al., 2000). Moreover, the thymus is one of the body tissues with the highest content of GC receptors

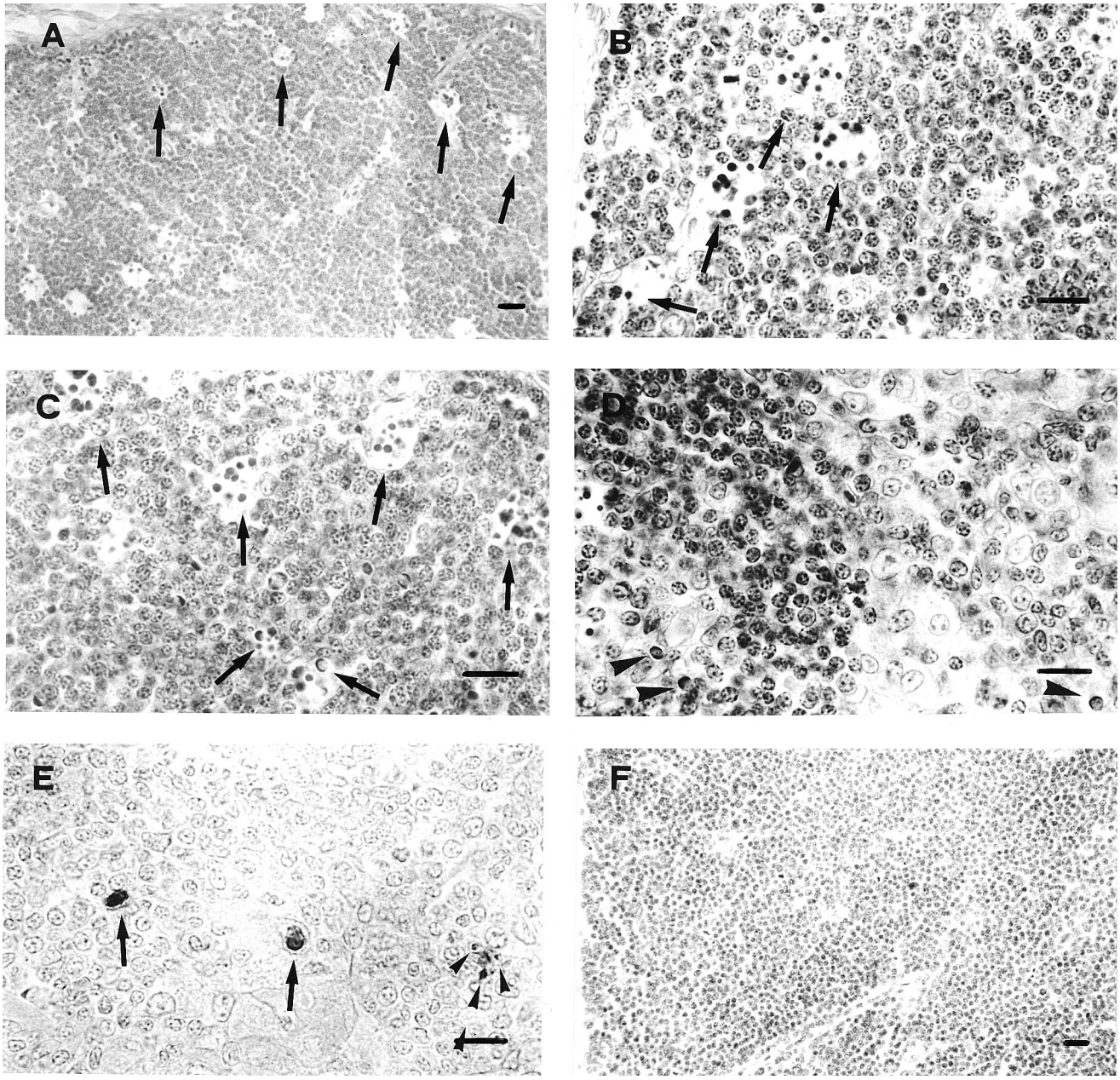
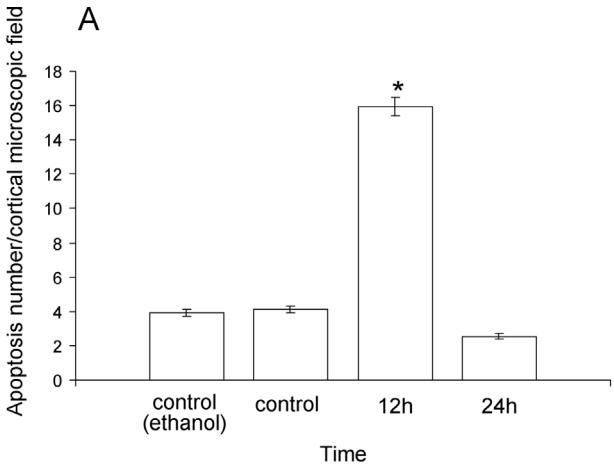
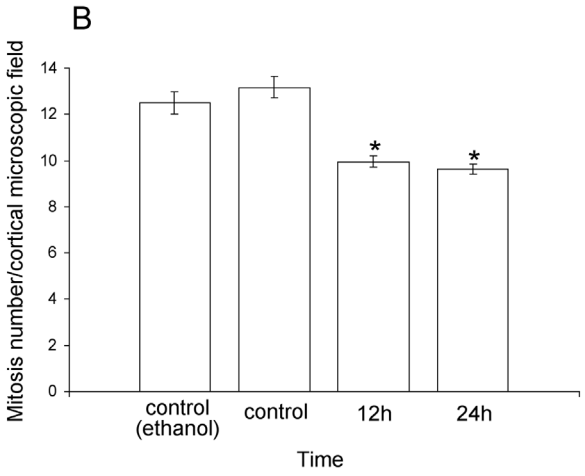


Fig. 1. Thymic sections from 21-day-old chickens 12 h **(A-E)** and 24 h **(F)** after corticosterone 21-acetate (20 mg/Kg) treatment stained with hematoxylin and eosin **(A-D, F)** and anti-caspase-3 antibody **(E)**. Groups of cells with an apoptotic phenotype (arrows) are present in the outer **(A, B)** and inner cortex **(C)**, while in the medulla a lower number of isolated apoptotic cells (arrowheads) is seen (**D**). Nuclei (arrows) and nuclear fragments (arrowheads) positive to anti-caspase-3 antibody are shown in the cortical-medullar region **(E)**. Bar: 10 μm.





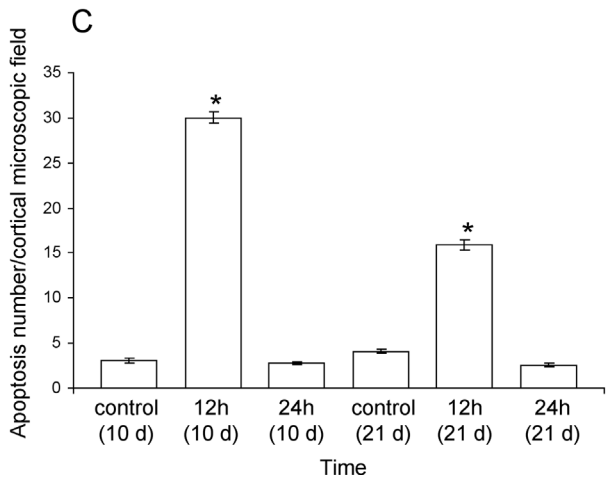
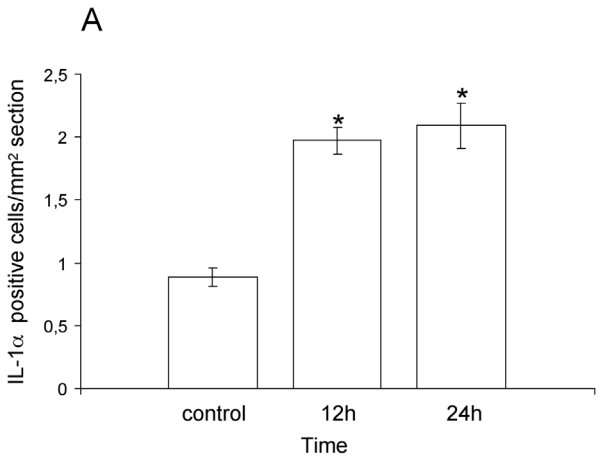
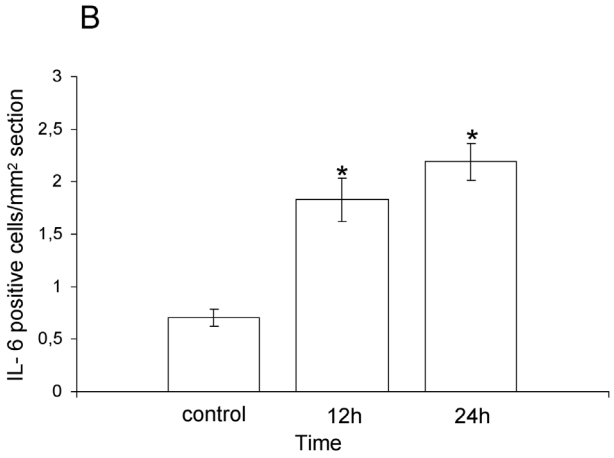


Fig. 2. Statistical analysis of cell number changes in thymic sections of 21-day-old chickens after corticosterone 21-acetate (20 mg/Kg) treatment: apoptosis **(A, C)**; mitosis **(B)**. The difference in the number of apoptotic cells between differently aged chickens is compared in **(C)**. d: day from hatching. *: P<0.05 vs control.





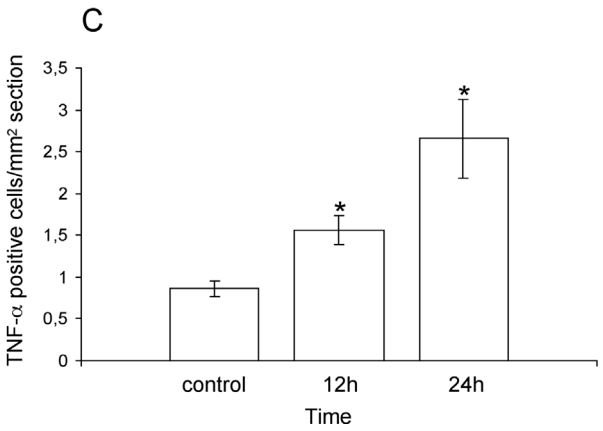


Fig. 3. Statistical analysis of cell number changes in thymic sections of 21-day-old chickens after corticosterone 21-acetate (20 mg/Kg) treatment: cells immunoreactive to anti-cytokine antibodies **(A-C)**. *: P<0.05 vs control.

(Miller et al., 1990) and in human thymus, immature thymocytes show a higher receptor density (Ranelletti et al., 1987).

The observed reduction in thymic cell population after 12 h of corticosterone treatment is not only due to an increased number of cells involved in apoptosis, but also to reduced mitotic activity. Moreover, younger animals appear to be more sensitive, as a higher number of cell deaths was observed.

As far as the presence of cytokine-like molecules is concerned, the acute stress stimulated molecule expression in our model. After 12 h of treatment (20 mg/Kg), the 21-day-old chicken thymus showed an increased number of cells containing immunoreactive molecules to anti-IL1-α, IL6 and TNF-α antibodies and these cells were located in the medulla and the thymic region, where the thymocytes seem to be resistant to GC-induced apoptosis. These results suggest an inhibitory effect of cytokine-related molecules on apoptosis as previously found (Kam et al., 1993; Mor and Cohen, 1996). Many studies demonstrated the inhibitory effects of GCs on the production and release of numerous cytokines (Munck et al., 1984; Munck and Naray-Fejes-Toth, 1994; Almawi et al., 1996), even if not all are suppressed by GCs (Munck and Naray-Fejes-Toth, 1994; Wiegers and Reul, 1998). On the other hand, various experimental designs paradoxally reported the synergism between GCs and cytokines on several cell types, probably by the action of cytokines, i.e. TNF-α and IL1ß, on the activity of enzymes present in lymphoid organ stromal cells that regulate the active GC concentration (Wiegers and Reul, 1998).

Oldenburg et al. (1997) demonstrated that *in vivo* dexamethasone resistance of a small population of mature thymocytes seems to be due to endogenous thymic soluble factors (i.e. cytokines) able to alter receptor signalling, rather than to the alteration of the GC receptor or to the expression of apoptosis inhibitors such as Bcl-2. Moreover, pro-inflammatory cytokines, such as TNF-α and IL-1, are able to regulate human GC-receptor gene expression by selective accumulation of the ß isoform receptor that attenuates the action of GC binding α isoform, explaining the generation of GC resistance (Webster et al., 2001). The presence of

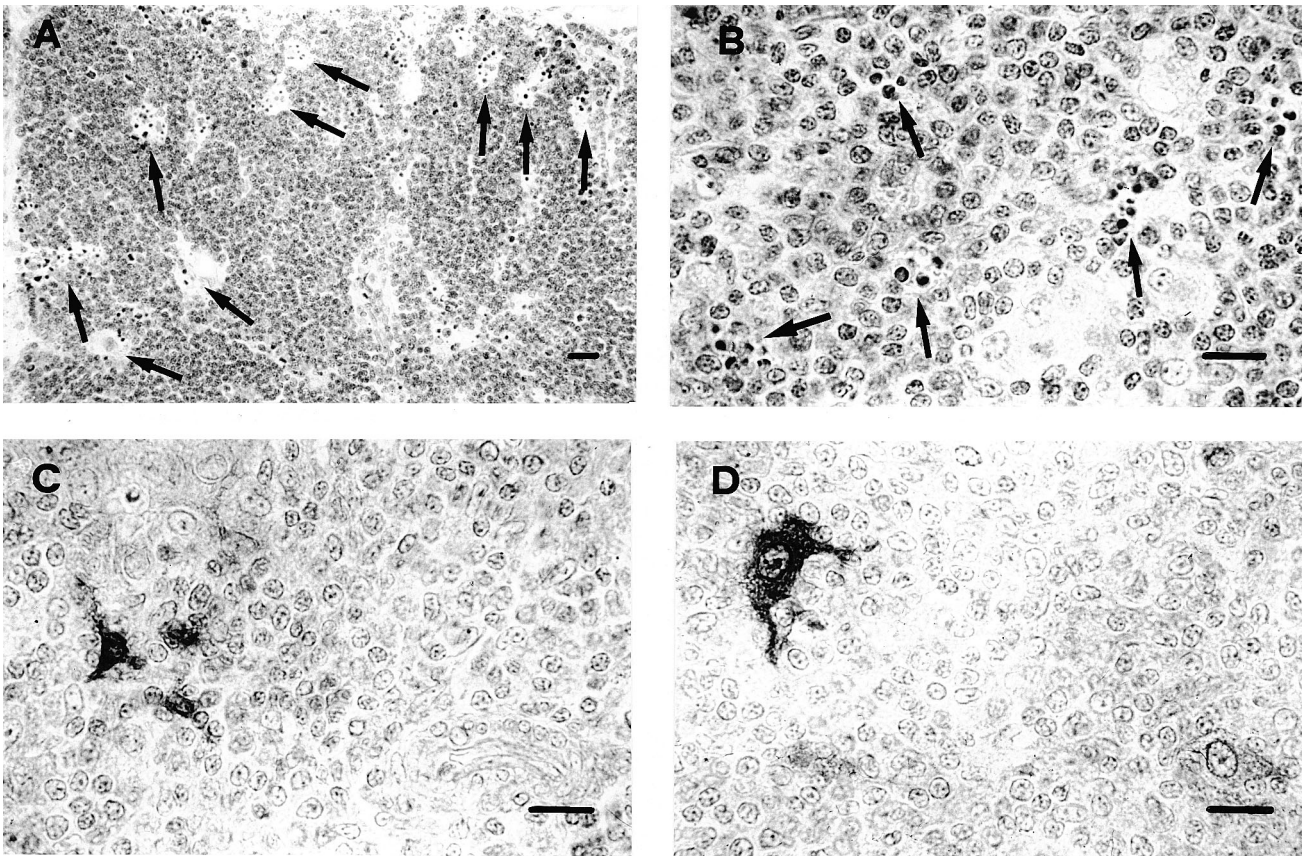


Fig. 4. 12 h after corticosterone 21-acetate (20 mg/Kg) treatment of 10-day-old chickens, the groups of apoptotic cells (arrows) present in the cortex **(A)** and the medulla **(B)** increase in comparison to older animals. Thymic sections stained with hematoxylin and eosin. Medullar epithelial cells positive to anti-TNF-α antibody **(C)** and an interdigitating cell in cortical-medullar region positive to anti-IL-1α antibody **(D)** are shown in a control section of 21-day-old chickens. Bar: 10 μm.

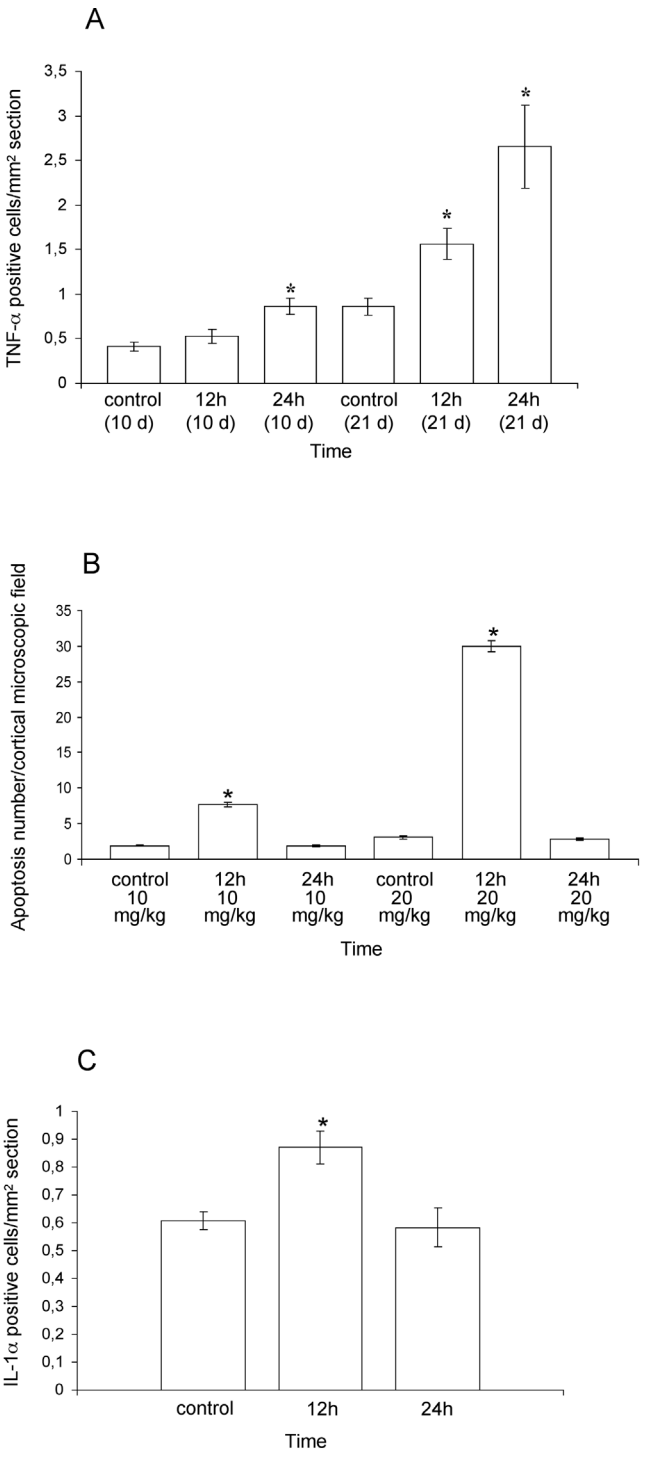


Fig. 5. Comparative analysis of the number of cells immunoreactive to the anti-TNF-α antibody after corticosterone 21-acetate (20 mg/Kg) treatment of 10- and 21-day-old chickens **(A)** and the number of apoptosis after injection of 10 and 20 mg/Kg in 10-day-old chickens **(B)**. Analysis of cells positive to anti-IL-1α antibody after a 10 mg/Kg corticosterone treatment of 10-day-old chickens **(C).** d:day from hatching. * P: <0.05 vs control.

cytokines in interdigitating cells, a specialized type of macrophage of the cortical-medullary region (Duijvestijn et al., 1982), could indicate a possible role of these molecules in the terminal step of the apoptotic process, i.e. phagocytosis. Indeed, cytokines have been seen to increase bacterial phagocytic activity (Ottaviani et al., 1997b) and apoptotic material has been found inside the cytoplasm of thymic interdigitating cells of *G. domesticus* (Ottaviani et al., 1997a). Furthermore, studies on the thymus from birds of different ages have revealed that cytokine-like molecules are present in microenvironment cells soon after birth, that they increase during age-related organ development and that they are still present in the involution phase, suggesting an important role in thymic physiology (Lenardo, 1991). Another interesting observation that emerges from the present study is that younger animals, which are more sensitive to GC treatment, show a reduced cytokine response.

On the whole, we can conclude that cytokines activated by acute stress *in vivo* could, probably together with other factors, contribute to restoring immunological homeostasis and influence thymic GC-mediated functions. This is in agreement with the new view of adrenal steroid effects on immune function as modulators of the immune system in contrast to the more traditional one that considered GC immunesuppressor factors, as proposed by McEwen et al. (1997).

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