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Immunohistochemical Changes in The Thyroid and Adrenal Glands Under the Influence of Mycobacteria: An Age-Dependent Experimental Study (CASPASE-3, CD68, P53 AND INHIBIN)

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Abstract

Background and objective. Chronic inflammation induced by mycobacteria-containing Freund's adjuvant may alter apoptosis, macrophage activity and the functional state of endocrine cells. This study evaluated the age-dependent immunohistochemical (IHC) changes of the thyroid and adrenal glands in rats exposed to mycobacteria, using Caspase-3 and CD68 (thyroid) and p53 and inhibin (adrenal).

Methods. Outbred white male rats aged 4 and 9 months were studied; experimental animals received a single subcutaneous injection of Freund's adjuvant containing killed mycobacteria, and intact animals served as controls. IHC was performed with DAB chromogen; slides were scanned and the positive-expression level (%) was quantified with QuPath-0.5.1 at ×200 magnification.

Results. In the thyroid, mycobacterial exposure markedly increased Caspase-3 expression (from 10.57% to 51.11% at 4 months and from 9.23% to 75.00% at 9 months) and CD68 expression (from 15.43% to 30.37% and from 16.98% to 31.71%, respectively). In the adrenal cortex, p53 expression

decreased (from 48.51% to 29.96% and from 45.77% to 21.75%) and inhibin expression decreased (from 42.08% to 25.31% and from 39.05% to 29.50%). Conclusion. Mycobacteria induce a reproducible, age-modified IHC signature — enhanced thyroid apoptosis (Caspase-3) and macrophage infiltration (CD68) together with reduced adrenal p53 and inhibin expression — reflecting parallel immune-inflammatory injury of the thyroid and functional impairment of adrenocortical cells

Conclusion. Pulmonary morphology in these poisonings is corroborative rather than diagnostic. A defensible medico-legal conclusion integrates scene investigation, autopsy, histology and—decisively—toxicology (ethanol concentration and COHb). Awareness of the combined-exposure scenario and of analytical pitfalls is critical to avoid misclassification.

Keywords: Immunohistochemistry; Caspase-3; CD68; p53; inhibin; thyroid gland; adrenal gland; mycobacteria; QuPath; experimental morphology.

Introduction

Chronic and autoimmune inflammatory disorders of the endocrine glands are associated with altered cellular turnover, immune-cell infiltration and impaired secretory function [1, 2]. Mycobacteria-containing complete Freund's adjuvant is a classical trigger of chronic systemic immune inflammation and is widely used to reproduce autoimmune-type organ injury; its effect is driven by mycobacterial cell-wall molecules that activate innate immunity and macrophages [3, 4, 25]. Immunohistochemistry (IHC) allows the objective, quantitative assessment of the molecular processes underlying such injury.

In the thyroid gland, apoptosis of follicular epithelial cells is a key mechanism of parenchymal loss in autoimmune thyroiditis, and increased Caspase-3 expression accompanies this process [5, 6, 7]. Macrophages, identified by the pan-macrophage marker CD68, are central effectors of the mononuclear-phagocyte response and their density reflects the intensity of the local inflammatory reaction [8, 9, 12]. In the adrenal cortex, the tumour-suppressor protein p53 participates in the control of cell-cycle arrest, apoptosis and the DNA-damage response, and is also linked to the regulation of steroidogenesis [13, 14, 15], whereas the inhibin α-subunit is a marker of differentiated steroidogenic adrenocortical cells whose expression reflects their functional state [16, 17, 18].

Although the individual roles of these markers are known, their combined, age-dependent behaviour in the thyroid and adrenal glands under mycobacterial exposure has not been well characterised. The objective of this study was to evaluate, by quantitative IHC, the age-dependent changes of Caspase-3 and CD68 in the thyroid and of p53 and inhibin in the adrenal gland of rats exposed to mycobacteria.

Methods

Animals and experimental groups

The study was performed on outbred white male rats of two age periods — 4 and 9 months. Four groups were formed: control-1 (intact, 4 months), experimental-1 (mycobacteria, 4 months), control-2 (intact, 9 months) and experimental-2 (mycobacteria, 9 months). Group size was substantiated using the resource-equation and power-analysis approaches [22]. Chronic immune-inflammatory injury was reproduced by a single subcutaneous injection of Freund's adjuvant (0.2 mL) containing killed Mycobacterium tuberculosis (H37Ra) into the sacral region; intact animals of the corresponding age served as controls [3, 21]. The observation period was one month. All procedures complied with good laboratory practice and Directive 2010/63/EU [23, 24].

Immunohistochemistry and quantification

Thyroid and adrenal tissues were fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned at 4–5 µm. IHC was performed with primary antibodies against Caspase-3 and CD68 (thyroid) and p53 and inhibin (adrenal), using 3,3′-diaminobenzidine (DAB) as chromogen; expressing cells appeared brown/red. Stained sections were digitised at ×200 magnification and analysed with QuPath-0.5.1 software, which determined the positive-expression level as the percentage of positively stained area/cells. Distribution was checked with the Shapiro–Wilk test; comparisons used Student's t-test or the Mann–Whitney U test, with $p < 0.05$ considered significant.

Results

Thyroid apoptosis (Caspase-3)

In intact animals, Caspase-3 expression in the thyroid was low (10.57% at 4 months and 9.23% at 9 months), consistent with a physiological rate of follicular-cell turnover. Under mycobacterial exposure, Caspase-3 expression increased sharply, reaching 51.11% at 4 months and 75.00% at 9 months, indicating intense activation of apoptosis in follicular and parafollicular cells; the increase was most pronounced in the older animals (Table 1; Figure 1).

Thyroid macrophages (CD68)

CD68 expression in intact thyroid was moderate and stable (15.43% at 4 months and 16.98% at 9 months). Mycobacterial exposure approximately doubled CD68 expression (30.37% at 4 months and 31.71% at 9 months), reflecting marked macrophage infiltration of the interstitium and perifollicular zones (Table 1; Figure 2).

Table 1. Positive immunohistochemical expression (%) of Caspase-3 and CD68 in the thyroid gland (QuPath-0.5.1, ×200)

Marker	Control, 4 mo	FA, 4 mo	Control, 9 mo	FA, 9 mo
Caspase-3	10.57	51.11	9.23	75.00
CD68	15.43	30.37	16.98	31.71

Note. Values are the positive-expression level (% of stained area/cells) measured with QuPath-0.5.1. FA — Freund's adjuvant (mycobacteria); mo — months. Differences vs the corresponding control are statistically significant ($p < 0.05$).

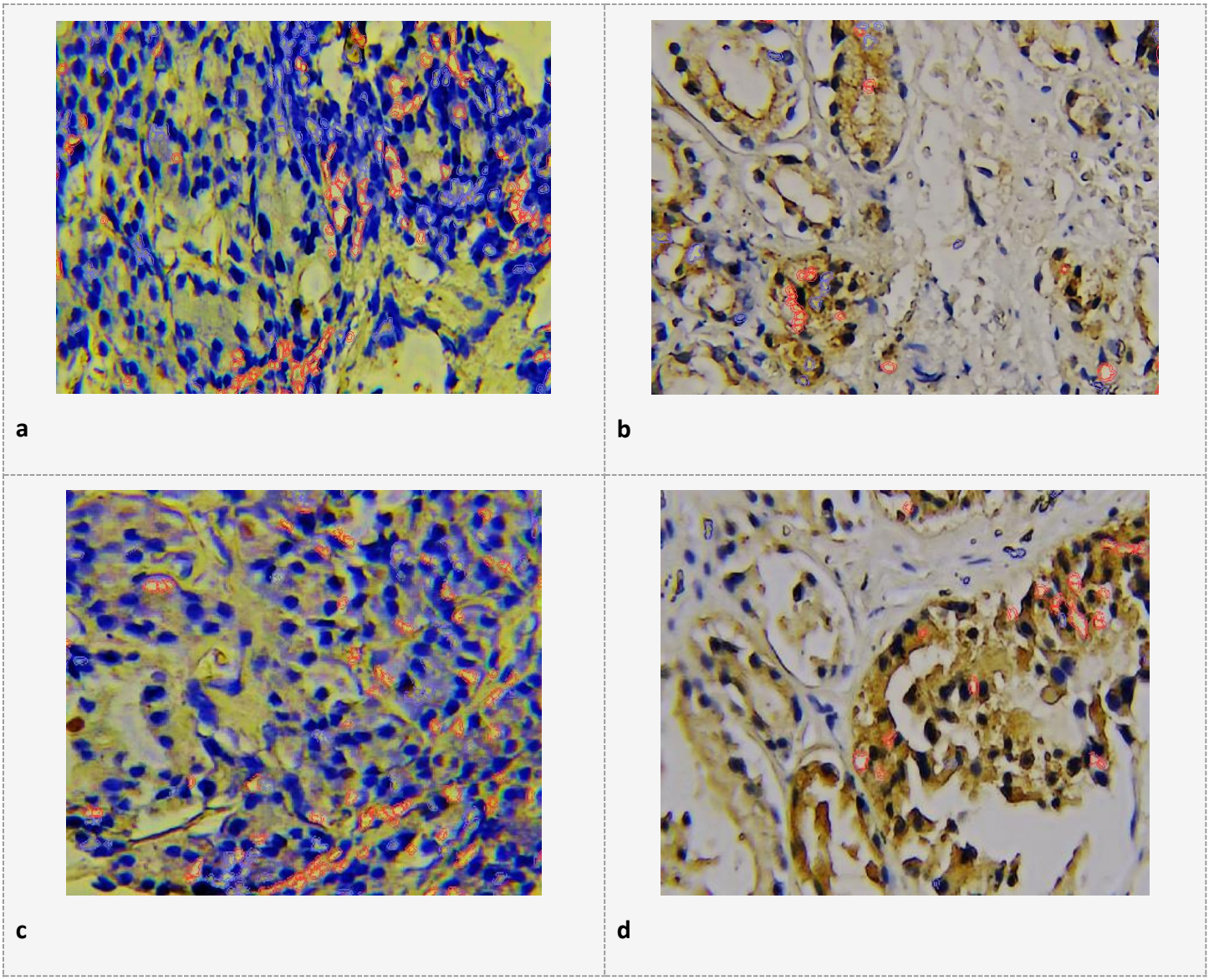

Figure 1. Immunohistochemical expression of Caspase-3 in the thyroid gland (DAB chromogen, ×200; QuPath-0.5.1). (a, c) intact controls — low expression; (b, d) mycobacteria-exposed animals — markedly increased expression, most pronounced at 9 months. Scale bar = 50 µm.

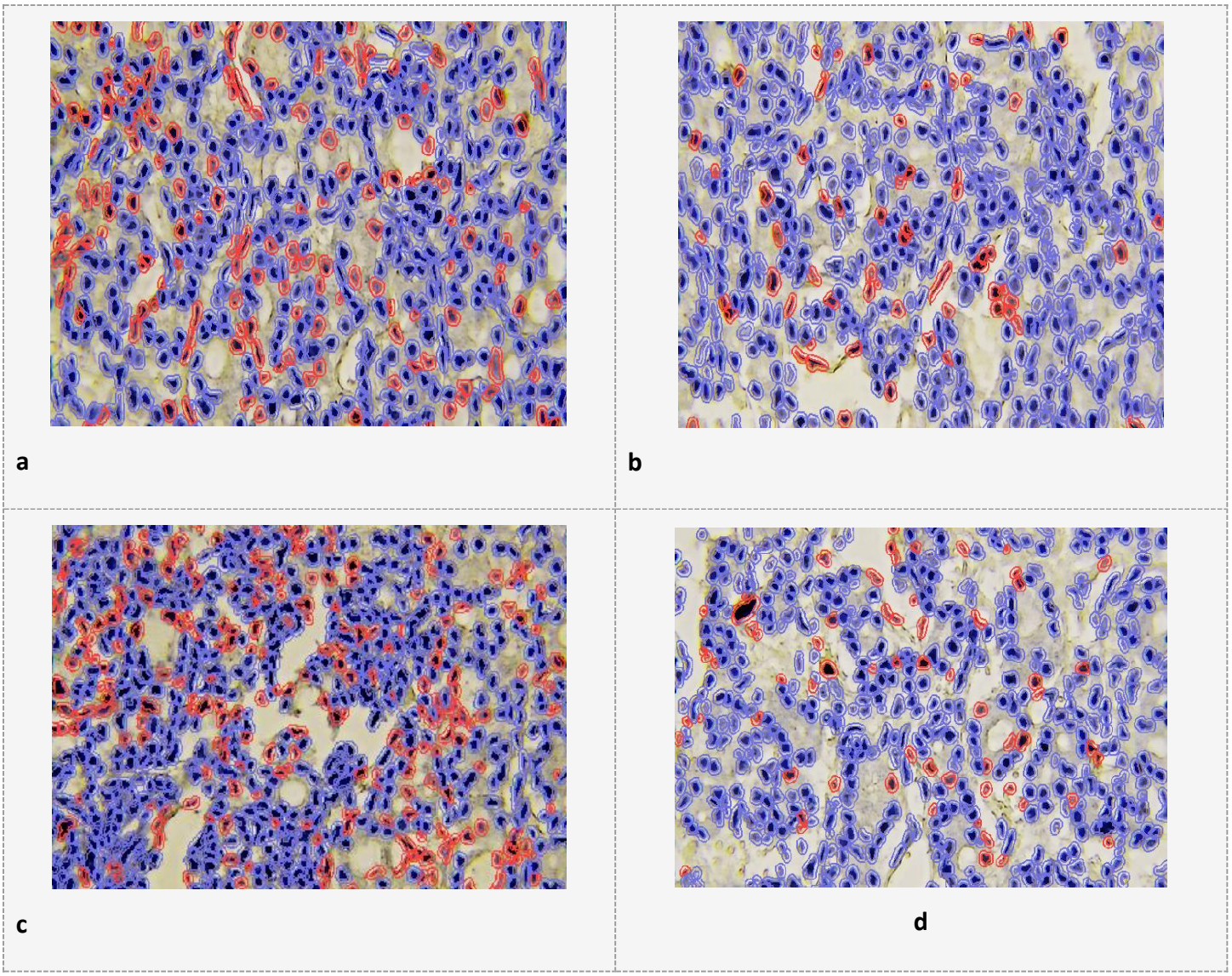

Figure 2. Immunohistochemical expression of CD68 (macrophages) in the thyroid gland (DAB chromogen, ×200; QuPath-0.5.1). (a, c) intact controls — moderate expression; (b, d) mycobacteria-exposed animals — increased macrophage infiltration. Scale bar = 50 µm.

Adrenal p53

In the intact adrenal cortex, p53 expression was relatively high (48.51% at 4 months and 45.77% at 9 months), reflecting the baseline participation of p53 in cellular homeostasis. Under mycobacterial exposure, p53 expression decreased to 29.96% at 4 months and 21.75% at 9 months, the reduction being greater in the older animals (Table 2; Figure 3).

Adrenal inhibin

Inhibin expression in the intact adrenal cortex was moderate to high (42.08% at 4 months and 39.05% at 9 months), in keeping with its role as a marker of differentiated steroidogenic corticocytes. Mycobacterial exposure reduced inhibin expression to 25.31% at 4 months and 29.50% at 9 months, indicating impaired functional differentiation of adrenocortical cells (Table 2; Figure 4).

Table 2. Positive immunohistochemical expression (%) of p53 and inhibin in the adrenal gland (QuPath-0.5.1, ×200)

Marker	Control, 4 mo	FA, 4 mo	Control, 9 mo	FA, 9 mo
p53	48.51	29.96	45.77	21.75
Inhibin	42.08	25.31	39.05	29.50

Note. Values are the positive-expression level (% of stained area/cells) measured with QuPath-0.5.1. FA — Freund's adjuvant (mycobacteria); mo — months. Differences vs the corresponding control are statistically significant ($p < 0.05$).

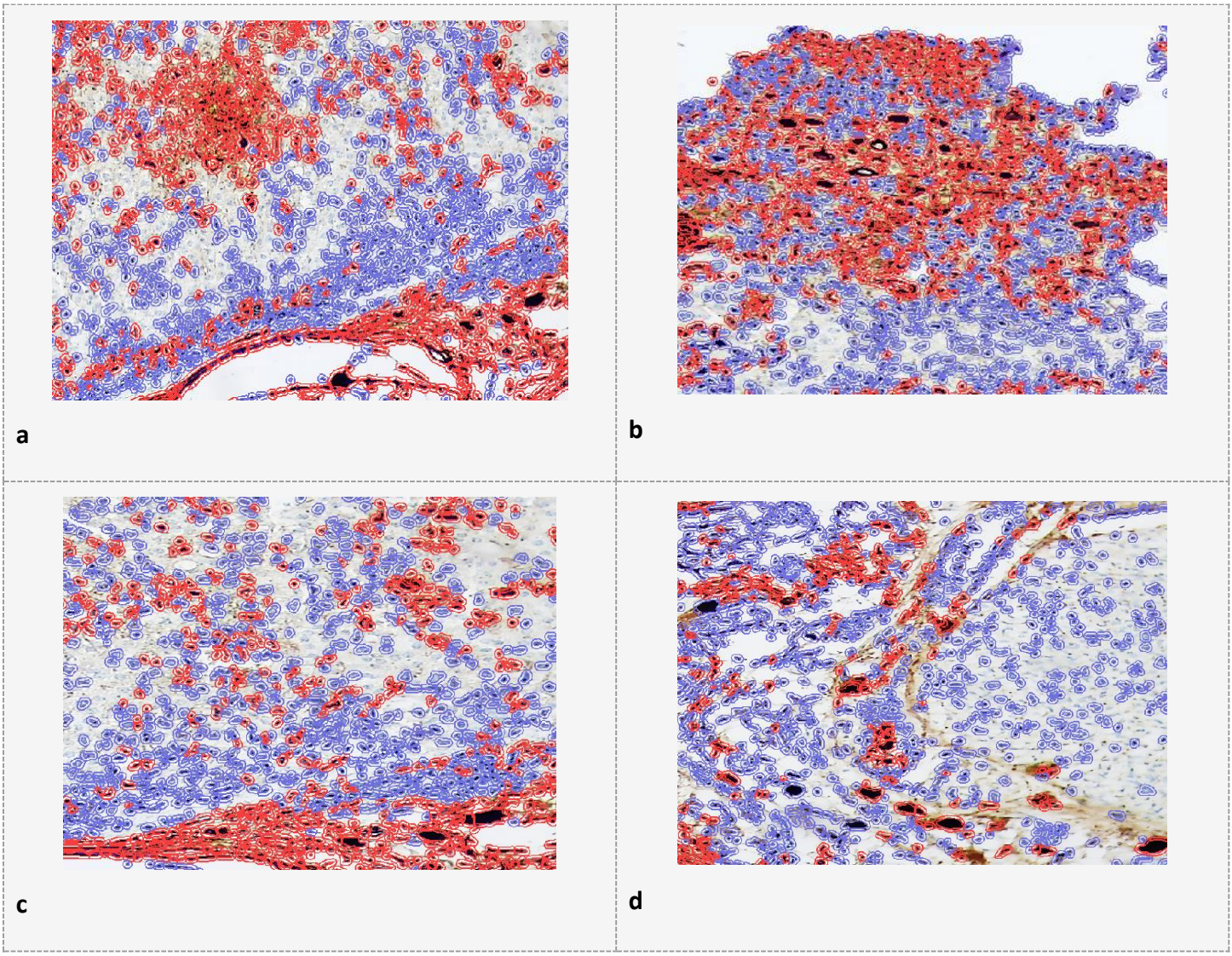

Figure 3. Immunohistochemical expression of p53 in the adrenal cortex (DAB chromogen, ×200; QuPath-0.5.1). (a, c) intact controls — higher expression; (b, d) mycobacteria-exposed animals — reduced expression, most marked at 9 months. Scale bar = 50 µm.

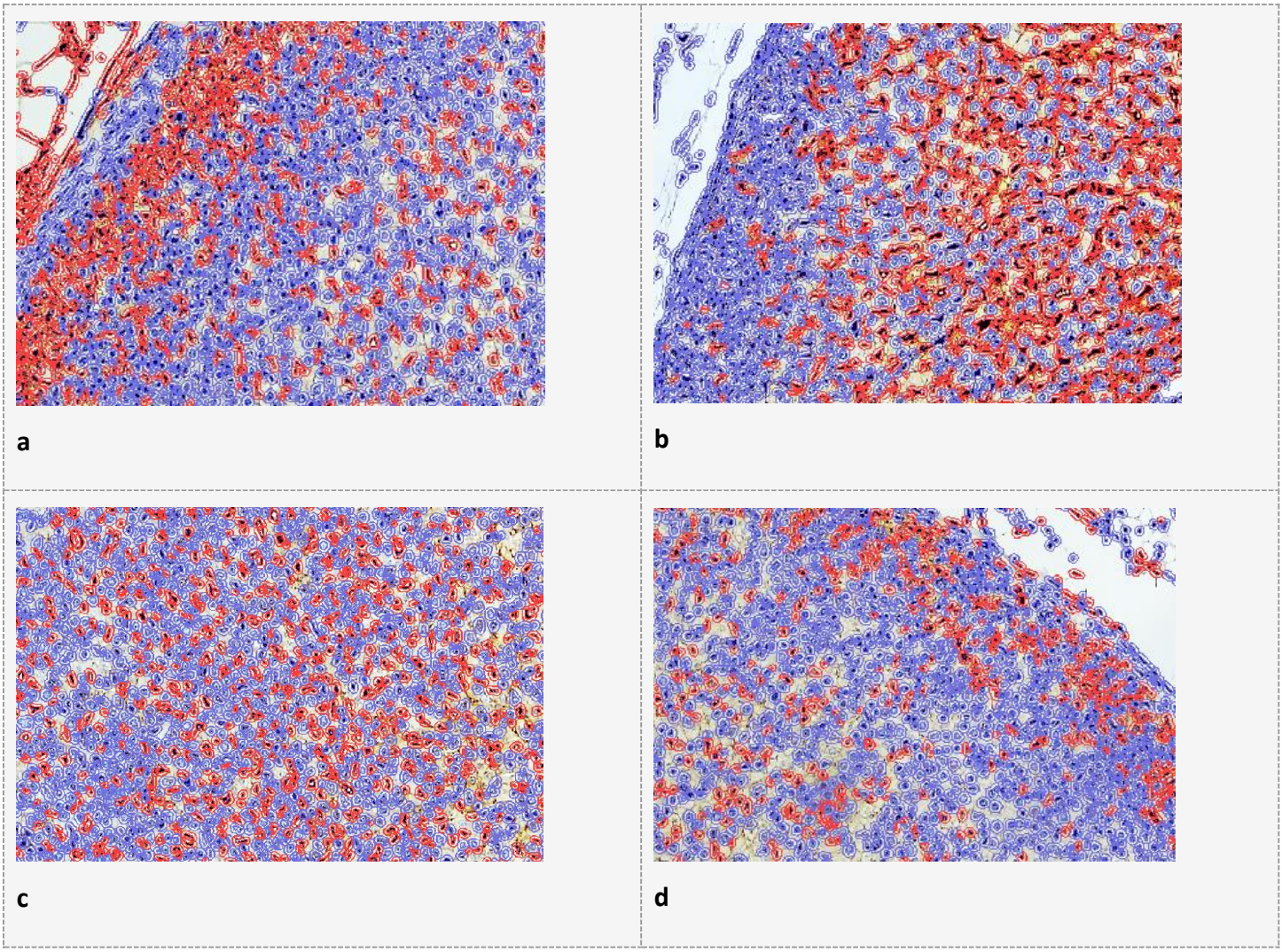

Figure 4. Immunohistochemical expression of inhibin in the adrenal cortex (DAB chromogen, ×200; QuPath-0.5.1). (a, c) intact controls — moderate-to-high expression; (b, d) mycobacteria-exposed animals — reduced expression. Scale bar = 50 µm.

Quantitative summary

The quantitative comparison of all four markers is summarised graphically in Figures 5 and 6. The thyroid markers (Caspase-3, CD68) increased under mycobacterial exposure, whereas the adrenal markers (p53, inhibin) decreased, at both ages.

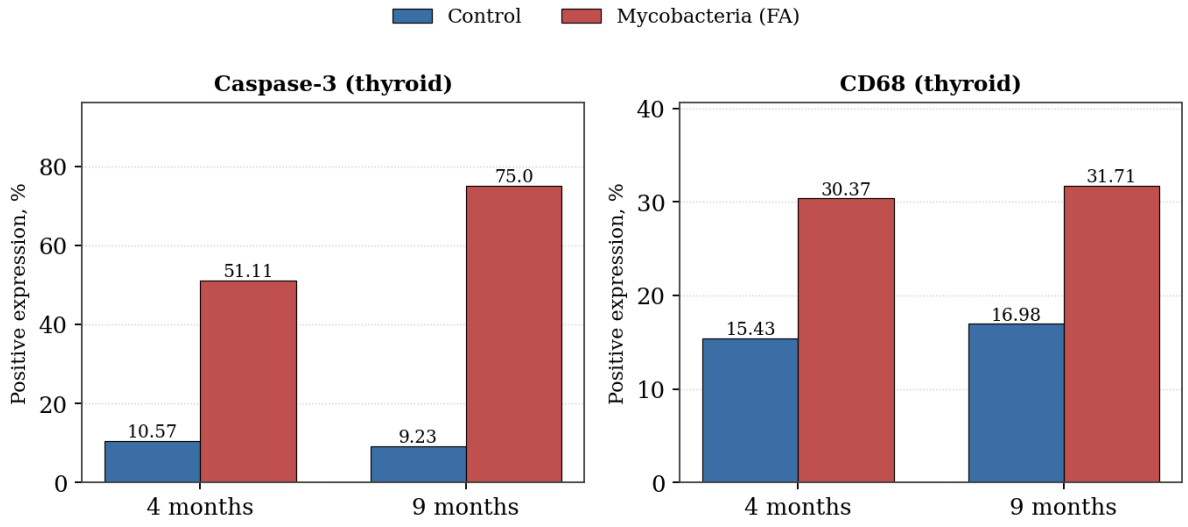

Figure 5. Positive immunohistochemical expression (%) of Caspase-3 and CD68 in the thyroid gland in control and mycobacteria (FA) groups at 4 and 9 months (QuPath-0.5.1, ×200).

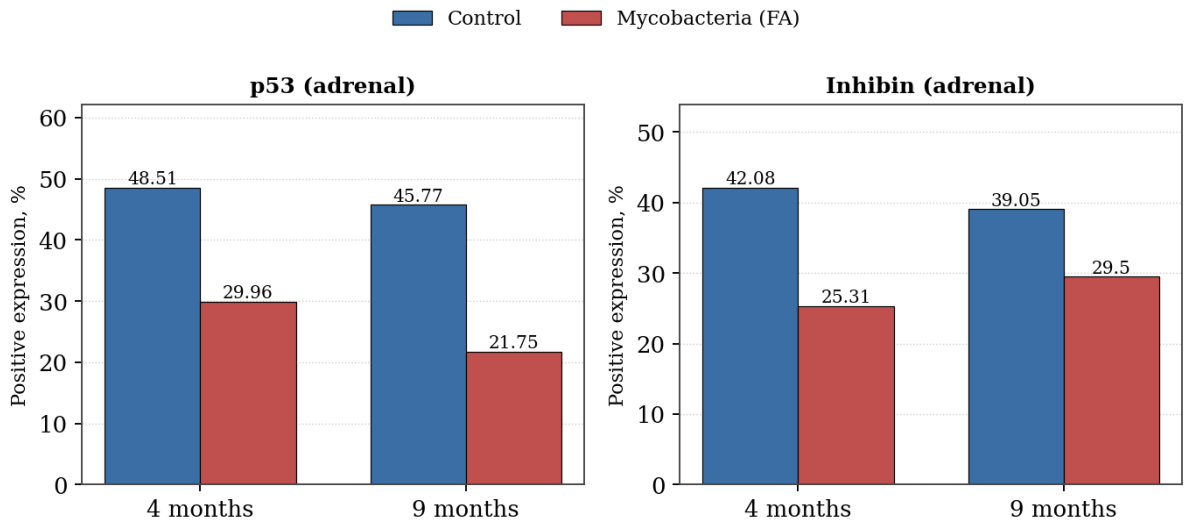

Figure 6. Positive immunohistochemical expression (%) of p53 and inhibin in the adrenal gland in control and mycobacteria (FA) groups at 4 and 9 months (QuPath-0.5.1, ×200).

Discussion

This study demonstrates that mycobacterial exposure produces a distinct, age-dependent immunohistochemical signature in the thyroid and adrenal glands, with opposite directions of change in the two organs. In the thyroid, the sharp increase in Caspase-3 expression indicates intense activation of apoptosis in follicular cells. This is consistent with reports that apoptosis of thyroid follicular epithelial cells, accompanied by increased Caspase-3 levels, is a key mechanism of parenchymal damage in autoimmune thyroiditis [5, 6]. The magnitude of the increase, greatest at 9 months (75%), parallels the morphological follicular atrophy and colloid resorption characteristic of the model and supports apoptosis as a driver of the decline in functional thyroid tissue [7, 10].

The increase in CD68 expression reflects marked macrophage infiltration of the gland. Macrophages are central to the mononuclear-phagocyte response in autoimmune thyroid disease and participate in both tissue damage and remodelling [8, 9]; the doubling of CD68 expression thus provides an immunohistochemical correlate of the interstitial and

perifollicular inflammatory infiltrate. Together, elevated Caspase-3 and CD68 depict a combined apoptotic and inflammatory injury of the thyroid parenchyma.

In the adrenal cortex, both p53 and inhibin expression decreased under mycobacterial exposure. p53 is a key regulator of the cellular stress response, cell-cycle arrest and apoptosis, and is also linked to the control of steroidogenesis in adrenocortical cells [13, 15]; its reduction may reflect a disturbed cellular stress-response and homeostatic control under sustained inflammatory stress. Inhibin α-subunit is a recognised marker of differentiated, functionally active steroidogenic adrenocortical cells [16, 17, 18]; the decrease in its expression indicates impaired functional differentiation of corticocytes, in line with the lipid depletion and fatty dystrophy of the zona fasciculata described in this model. The greater reduction of adrenal marker expression, and the greater increase of thyroid Caspase-3, at 9 months indicate that the older gland is more vulnerable to sustained mycobacterial inflammation, consistent with a shift from compensation towards decompensation with age [19, 20, 21].

The study has limitations. IHC quantification is based on the positive-expression level measured with QuPath, reported here as single group values; exact per-animal statistics and antibody clone/dilution details should accompany the full dataset. The findings are descriptive-quantitative and should be integrated with the morphometric and hormonal data of the model. Nonetheless, the concordant, biologically coherent changes across four markers strengthen the interpretation. Adherence to reporting and welfare guidelines supports reproducibility [23, 24].

Conclusion

Mycobacterial exposure induces a reproducible, age-modified immunohistochemical signature in the endocrine glands: in the thyroid, markedly increased Caspase-3 (apoptosis) and CD68 (macrophage) expression, and in the adrenal cortex, decreased p53 and inhibin expression. This pattern reflects parallel apoptotic-inflammatory injury of the thyroid and functional impairment of adrenocortical cells, both more pronounced at the older age. The quantitative IHC data complement the morphological and morphometric characterisation of the model and provide a basis for further functional and molecular investigation.

Authors' contributions

B.A. S. conceived and designed the study, performed the histological and immunohistochemical analyses and QuPath quantification, interpreted the data, and wrote and approved the final manuscript. T.S.J. supervised the immunohistochemical work, performed the digital image analysis, and reviewed and edited the manuscript. All authors have read and approved the final version of the manuscript and agree to be accountable for all aspects of the work.

Declarations

Ethics approval. All animal procedures were conducted in accordance with good laboratory practice and Directive 2010/63/EU and approved by the local bioethics committee. Approved by the Local Ethics Committee of the Bukhara State Medical Institute named after Abu Ali ibn Sino

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Conflict of interest. The author declares no conflict of interest.

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