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1 **Gene expression profile associated with thymus regeneration in dexamethasone-**
2 **treated beef cattle**

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4 F.T. Cannizzo, L. Starvaggi Cucuzza*, S. Divari, E. Berio, F.E. Scaglione, B. Biolatti

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6 *Dipartimento di Scienze Veterinarie, University of Turin, Largo Paolo Braccini 2, 10095*

7 *Grugliasco (TO), Italy*

8

9 *Corresponding author. Tel. +39 011 670 9037;

10 Fax +39 011 6703031

11 E-mail address: laura.starvaggicucuzza@unito.it (L. Starvaggi Cucuzza)

12

13 **Abstract**

14 Glucocorticoids are illegally used as growth promoters in cattle and the analytical methods
15 officially applied most likely underestimate the precise frequency of the abuse. As a side
16 effect, the administration of glucocorticoids causes fat infiltration, apoptosis and atrophy of
17 the thymus. However, gross and histological observations carried out previously showed
18 that the thymus preserves an intrinsic ability to regenerate. The aim of this work was to
19 study the transcriptional effects of glucocorticoids on genes likely involved in regeneration
20 of the epithelial cell network in cervical and thoracic thymus of beef cattle treated with
21 dexamethasone or prednisolone in comparison with the control group. Moreover, the ratio
22 of BAX/BCL2 genes was examined to verify a possible anti-apoptotic activity occurring at
23 the same time. In cervical thymus, dexamethasone administration increased the gene
24 expression of c-Myc ($P < 0.01$), TCF3 ($P < 0.05$), TP63 ($P < 0.01$) and KRT5 ($P < 0.01$). In
25 thoracic thymus of dexamethasone-treated cattle the gene expression of TCF3 ($P < 0.01$),
26 TP63 ($P < 0.01$) and KRT5 ($P < 0.05$) was increased. These results suggested that thymic
27 regeneration is underway in the dexamethasone-treated animals. However, the BAX/BCL2
28 ratio was decreased in both cervical and thoracic thymus of dexamethasone-treated cattle
29 ($P < 0.01$ and $P < 0.05$, respectively), showing an anti-apoptotic effect through the
30 mitochondrial pathway. Conversely, prednisolone administration caused no change in the
31 expression of all considered genes. These results sustain the hypothesis that regeneration
32 occurs in the thymus parenchyma 6 days after the dexamethasone treatment was
33 discontinued. This hypothesis is also supported by the absence of alterations in thymus of
34 prednisolone-treated beef cattle. Indeed, previous studies showed the inability of
35 prednisolone to induce macro- and microscopical lesions in the thymus. Therefore, in this
36 context, it is not surprising that prednisolone induced no alteration of genes involved in
37 regeneration pathway.

38

39 **Keywords:** beef cattle;dexamethasone; glucocorticoids; prednisolone; regeneration;
40 thymus

41 **1. Introduction**

42 The administration of natural and synthetic hormones as growth promoters in animals is
43 banned in the European Union (EU) and analytical methods are officially applied by
44 national surveillance programs to prevent the illegal use of hormones [1].

45 Dexamethasone (DEX) is one of the most commonly administered glucocorticoids (GCs),
46 and induces fat infiltration, increases apoptosis and causes atrophy of the thymus in cattle,
47 as a side effect [2-4]. Conversely, prednisolone (PRD), another illicitly used GC, seems to
48 be unable to induce thymus atrophy [5]. However, it is conceivable that the thymus
49 preserves an intrinsic ability to regenerate after GCs administration, because the bovine
50 thymic parenchyma and activity could be restored, as previously shown by gross and
51 histological observations [4]. Nevertheless, the mechanisms controlling thymus
52 regeneration remain largely unknown. Conversely, no cellular response seems to be
53 triggered by PRD administration. It was previously shown that some transcription factors
54 are over-expressed in the thymic stroma of mice [6]. The thymic epithelial cells (TECs)
55 showed an up-regulation of c-Myc, TCF3 and TP63 genes during DEX- or irradiation-
56 induced atrophy and a down-regulation after regeneration. These transcription factors
57 were previously shown to regulate differentiation of epithelial stem cells in various tissues
58 [7-9], suggesting a role in reconstruction/maintenance of the epithelial cell network.
59 Moreover, it has been demonstrated that DEX- and irradiation-induced damage of the
60 thymus resulted in proliferation of specific subset of TEC precursors expressing keratin 5
61 (KRT5) [6].

62 Nevertheless, tissue re-growth is not only the result of cell proliferation, but also of
63 enhanced cell survival by means of the inhibition of apoptosis or a combination of both
64 mechanisms [10]. Several studies have highlighted that many of the molecular pathways
65 involved in thymus atrophy rely on the mitochondria-dependent apoptotic pathway,
66 involving proteins of the BCL2 family [11,12]. The members of BCL2 family are known to

67 be key regulatory proteins in apoptotic events, and can promote either cell survival or cell
68 death. Indeed, the equilibrium between the pro- and anti-apoptotic members or their
69 relative amount are crucial to sensitise the cells towards either survival or apoptosis. The
70 anti-apoptotic effect of BCL2 acts by binding and inhibiting pro-apoptotic proteins like BAX.
71 The latter promotes apoptosis by altering mitochondrial functions and activating the
72 release of downstream apoptogenic factors [13]. The aim of this work was to study the
73 biological mechanisms involved in regeneration following GCs treatment.
74 Therefore, an increase of transcript abundance of c-Myc, TCF3, TP63, and KRT5 was
75 hypothesized in the cervical and thoracic thymus during regeneration of the thymus in beef cattle.
76 Additionally, the BAX and BCL2 expression and their ratio were examined to evaluate a
77 possible anti-apoptotic activity occurring at the same time.

78

79

80 **2. Material and methods**

81 *2.1. Animals*

82 The experiment was authorized by the Italian Ministry of Health and the Ethics Committee
83 of the University of Turin. The carcasses of the treated animals were appropriately
84 destroyed (2003/74/CE–DL 16 March 2006, No. 158).

85 All groups of experimental animals were kept in separate pens of 10 × 15 m and were fed
86 a diet consisting of corn silage, corn, hay and a commercial protein supplement; animals
87 had *ad libitum* access to water. Eighteen male Charolaise beef cattle of 17 to 22 mo of age
88 were divided into the following three groups: group A (n = 6) was administered
89 dexamethasone–21–sodium-phosphate 0.7 mg/d *per os* for 40 d; group B (n = 6) was
90 administered PRD 15 mg/d *per os* for 30 d; group K (n = 6) served as a control. Each
91 morning, before the distribution of feed, the animals were tied to the feed trough, and two
92 trained technicians administered orally one capsule containing the compound using a

93 drenching gun. The control animals were treated with a placebo. The animals were
94 slaughtered 6 d after drug withdrawal. The gross and microscopic findings in the thymus of
95 these animals were previously reported [5].

96

97 *2.2. Samples*

98 Thoracic and cervical thymus samples from each animal were collected and placed in
99 RNAlater solution (Ambion, Thermo Fisher Scientific, Waltham, MA) to preserve the RNA
100 integrity for molecular investigation.

101

102 *2.3. Total RNA extraction and quantitative PCR*

103 The expression of c-Myc, TCF3, TP63, KRT5, BAX and BCL2 in the thoracic and cervical
104 thymus was investigated by quantitative PCR (qPCR). For this purpose, fifty milligrams of
105 thymus were disrupted using a TissueLyser II (Qiagen, Hilden, Germany) with stainless
106 steel beads in 1 mL of TRIzol (Invitrogen, Thermo Fisher Scientific). Total RNA was
107 purified from any residual genomic DNA with a DNA-free kit (Ambion). The integrity of the
108 RNA was confirmed by the Experion Automated Electrophoresis Station (Bio-Rad,
109 Hercules, CA), and the concentration was measured by a spectrophotometry. cDNA was
110 synthesised from 1 µg of total RNA using ImProm-II reverse transcriptase (Promega,
111 Madison, WI) and random primers (Promega). To determine the amount of the specific
112 target genes, cDNA was subjected to qPCR using the SYBRGreen method and the IQ5
113 (Bio-Rad) detection system. The primer sequences were designed using Primer Express v
114 1.5 (Applied Biosystems, Thermo Fisher Scientific) (Table 1). The peptidylprolyl isomerase
115 A (cyclophilin A, PPIA) gene was used as a housekeeping control gene, as previously
116 reported [14]. The expression level of each target gene was calculated using the $2^{-\Delta Cq}$
117 method, where $\Delta Cq = Cq_{\text{target gene}} - Cq_{\text{housekeeping gene}}$ [15].

118

119 2.4. Statistical analysis

120 The data were analyzed using GraphPad InStat version 3.00 (GraphPad Inc., San Diego,
121 CA). The analysis of c-Myc, TCF3, TP63, KRT5, BAX and BCL2 gene expression and the
122 analysis of the ratio of BAX and BCL2 expression was performed using one-way analysis
123 of variance (ANOVA), followed by Dunnett's post hoc-test versus the control group K. If
124 Bartlett's test suggested that the difference between the standard deviations of each group
125 was significant, then the nonparametric Kruskal-Wallis test with Dunn's post-test versus
126 the control group was applied. The Grubbs test was used to reveal potential outliers. A *P*
127 value of < 0.05 was considered statistically significant. The data are shown as the mean
128 arbitrary units ($2^{-\Delta Cq}$) \pm SEM.

129

130

131 3. Results

132 In the cervical thymus, DEX administration (group A) increased c-Myc expression (mean of
133 mRNA arbitrary units \pm SEM: $9.46 \times 10^{-2} \pm 1.49 \times 10^{-2}$) compared with the control group K
134 ($4.70 \times 10^{-2} \pm 3.02 \times 10^{-3}$) ($P < 0.01$) (Fig. 1a), TCF3 expression ($8.74 \times 10^{-3} \pm 4.54 \times 10^{-3}$)
135 compared with the control group K ($8.48 \times 10^{-4} \pm 9.88 \times 10^{-5}$) ($P < 0.05$) (Fig. 1b), TP63
136 expression ($8.64 \times 10^{-2} \pm 2.71 \times 10^{-2}$) compared with the control group K ($1.56 \times 10^{-2} \pm$
137 1.85×10^{-3}) ($P < 0.01$) (Fig. 1c) and KRT5 expression ($2.31 \times 10^{-1} \pm 1.99 \times 10^{-2}$) compared
138 with the control group K ($3.75 \times 10^{-2} \pm 6.20 \times 10^{-3}$) ($P < 0.01$) (Fig. 1d). Conversely, DEX
139 administration decreased BAX expression ($1.54 \times 10^{-3} \pm 1.17 \times 10^{-4}$) compared with the
140 control group K ($3.44 \times 10^{-3} \pm 2.38 \times 10^{-4}$) ($P < 0.01$) (Fig. 2a). No change in BCL2
141 expression was observed (Fig. 2b). The administration of PRD (group B) caused no
142 change in the expression of all considered genes (Fig. 1 and 2).

143 In the thoracic thymus, DEX administration (group A) increased TCF3 expression ($5.68 \times$
144 $10^{-3} \pm 1.04 \times 10^{-3}$) compared with the control group K ($2.12 \times 10^{-3} \pm 2.51 \times 10^{-4}$) ($P < 0.01$)
145 (Fig. 1b), TP63 expression ($6.87 \times 10^{-2} \pm 8.70 \times 10^{-3}$) compared with the control group K
146 ($2.95 \times 10^{-2} \pm 3.62 \times 10^{-3}$) ($P < 0.01$) (Fig. 1c) and KRT5 expression ($1.79 \times 10^{-2} \pm 6.59 \times 10^{-3}$)
147 compared with the control group K ($5.37 \times 10^{-2} \pm 1.88 \times 10^{-3}$) ($P < 0.05$) (Fig. 1d). No
148 change in c-Myc (Fig. 1a) or BCL2 expression was observed (Fig. 2b). Conversely, DEX
149 administration decreased BAX expression ($3.20 \times 10^{-3} \pm 2.67 \times 10^{-4}$) compared with the
150 control group K ($4.91 \times 10^{-3} \pm 5.35 \times 10^{-4}$) ($P < 0.05$) (Fig. 2a). The administration of PRD
151 (group B) caused no change in the expression of all considered genes (Fig. 1 and 2).
152 The BAX/BCL2 ratio was statistically different in both cervical and thoracic thymus of
153 Group A compared to the controls ($P < 0.01$ and $P < 0.05$, respectively) (Fig. 2c).

154

155

156 **4. Discussion**

157 Council Directive 96/22/EC [1], as amended by Directives 2003/74/EC [16] and
158 2008/97/EC [17], stipulates that all use of steroids, β -agonists or other substances for the
159 chemical manipulation of animal growth is severely banned in the EU. However, results
160 from studies conducted by the Italian Health Ministry indicate that these substances are
161 persistently used, and therefore a permanent commitment by the public veterinary services
162 for their prevention and control is required. The thymus represents a GCs target tissue,
163 and in vitro or ex vivo qualitative and semi-quantitative morphological investigations to
164 identify the cellular effects of GCs were reported [2,4,18].

165 Indeed, the thymus weight and volume of beef cattle following long-term administration of
166 low doses of DEX were significantly reduced compared with the controls [4]. Moreover,
167 DEX-treated animals showed severe thymus atrophy, characterized by a serious volume

168 reduction of the organ, which in some animals almost disappeared and was replaced by
169 fat tissue. Histologically, the thymic cortex undergoes atrophy, while the medullary
170 framework was still present though reduced, showing a pronounced rarefaction of
171 lymphocytes [5]. Conversely, no histological change was observed in the thymus following
172 long-term treatment with PRD [5].

173 However, partial recovery of thymus weight and structure after 25 days followed by
174 complete recovery after 32 days was observed in veal calves [3] and similar results have
175 been observed in thymus of beef cattle examined 26 days after the end of treatment [4].

176 Thus, it is conceivable that the thymus preserves an intrinsic ability to regenerate, but the
177 molecular mechanisms controlling the regeneration of the thymus are largely unknown.

178 Previous work in mice demonstrated that c-Myc, TP63, and TCF3 gene expression was
179 up-regulated in TECs during peak thymic atrophy and was down-regulated at later time
180 points when thymuses were undergoing regeneration [6]. These transcription factors were
181 previously shown to regulate differentiation of epithelial stem cells in various tissues
182 [7,8,19] suggesting a role in reconstruction/maintenance of the epithelial cell network.

183 Consistent with these findings, our results showed an up-regulation of the same
184 transcription factors in thymus of beef cattle experimentally treated with a low doses of
185 DEX for a long-term. In contrast, the treatment with PRD did not induce any expression
186 changes in the genes examined in this study. Since the DEX administration induces the
187 thymus atrophy [4], whereas PRD treatment appears to have no effects on the thymus
188 tissue [5], the expression of the transcription factors might play a role in regeneration of
189 the thymic stroma.

190 Moreover, it has been demonstrated that DEX-induced damage of the thymus resulted in
191 proliferation of subset of TEC precursors expressing KRT5 [6]. This active expansion could
192 explain the significantly over-expression of KRT5 observed in the atrophic thymus of DEX-
193 treated beef cattle compared to controls.

194 Glucocorticoids influence the growth and differentiation of thymocytes through several
195 mechanisms including apoptosis [20]. The apoptotic effect of GCs could shift the balance
196 between expression of pro-survival and pro-apoptotic factors, ultimately leading to cell
197 death or apoptosis [21]. In this study, DEX administration caused a decrease of BAX
198 expression, whereas BCL2 expression remain unchanged. Moreover, the ratio of BAX and
199 BCL2 expression diminished. These results suggest that an anti-apoptotic effect is
200 occurring through the mitochondrial pathway, and this may support the hypothesis that
201 regeneration activity occurs in the thymus parenchyma 6 days after the treatment is
202 discontinued.

203 Thus, our data suggested that the observed recovery of thymus of DEX-treated beef cattle
204 might be mediated by several events, including the elevated expression of transcription
205 factors involved in differentiation of epithelial stem cells, active proliferation of TECs subset
206 and inhibition of apoptosis. The expression of this panel of genes appears characteristic of
207 the animals treated with DEX and not of PRD-treated animals. Indeed, previous studies
208 showed the inability of PRD to induce macro- and microscopical lesions (i.e. atrophy) in
209 thymus. Therefore, it is not surprising that PRD does not induce alteration of genes
210 involved in regeneration pathway.

211

212

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219

220

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307 **Table 1** Primer sequences used for qPCR.

Gene	Sense	Sequences 5' → 3'	Amplicon size (bp)	GeneBank accession number
c-Myc	Forward	ATGCCACGTGTCTACCCATCA	100	NM_001046074
	Reverse	GACCCTGCCACTGTCCAAC		
TCF3	Forward	TGGCTGAGTGCACCCTGAA	107	XM_002691408
	Reverse	CCGGGCCAATTCGTAGTACTT		
TP63	Forward	TTCCGTGAGCCAGCTTATCA	100	NM_001191337
	Reverse	GTGGGTGCCCATCATAGGAAT		
KRT5	Forward	GAGCCTTTGTTAGAGCAGTACATCAA	100	NM_001008663
	Reverse	CCTGCATATTCCTGAGCTCTGA		
BAX	Forward	AAGCGCATCGGAGATGAATT	100	NM_173894
	Reverse	CCGCCACTCGGAAAAAGAC		
BCL2	Forward	TGGTGGGCGCTTGCAT	100	NM_001166486
	Reverse	TTCTGCTGCTTCTTGAATCTTCTG		
PPIA	Forward	GCCCCAACACAAATGGTT	95	NM_178320
	Reverse	CCCTCTTTCACCTTGCCAAAG		

308

309 **Figure captions**

310 **Fig. 1.** Effects of dexamethasone-21-sodium phosphate (DEX; group A) or prednisolone
311 (PRD; group B) on c-Myc (a), TCF3 (b), TP63 (c) and KRT5 (d) gene expression
312 compared with the control group K in the cervical and thoracic thymus of beef cattle. The
313 results are presented as the means \pm SEM. The y-axes show arbitrary units representing
314 relative mRNA expression levels. * $P < 0.05$, ** $P < 0.01$ versus the control group K.

315

316 **Fig. 2.** Effects of dexamethasone-21-sodium phosphate (DEX; group A) or prednisolone
317 (PRD; group B) on BAX (a) and BCL2 (b) gene expression compared with the control group
318 K in the cervical and thoracic thymus of beef cattle. The results are presented as the means
319 \pm SEM. The y-axes show arbitrary units representing relative mRNA expression levels.
320 Effects of dexamethasone-21-sodium phosphate (DEX; group A) or prednisolone (PRD;
321 group B) on ratio of BAX and BCL2 expression (c) in cervical and thoracic thymus of beef
322 cattle. * $P < 0.05$, ** $P < 0.01$ versus the control group K.