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1 **Role of FKBP51 in the modulation of the expression of the corticosteroid receptors in**
2 **bovine thymus following glucocorticoid administration**

3

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13 **Abstract**

14 The aim of this work was to study the transcriptional effects of glucocorticoids on
15 corticosteroid hormone receptors, prereceptors (11 β -hydroxysteroid dehydrogenase 1 and 2,
16 11 β -HSD1 and 2) and chaperones molecules regulating intracellular trafficking of the
17 receptors (FKBP51 and FKBP52) in thymus of veal calves. Moreover, the expression of
18 FKBP51 and FKBP52 gene were investigated in beef cattle thymus. In the cervical thymus of
19 veal calves dexamethasone administration in combination with estradiol decreased FKBP51
20 expression ($P < 0.01$). The same treatment increased mineralocorticoid receptor (MR) ($P <$
21 0.01) and 11 β -HSD1 expression ($P < 0.05$) compared to control group in the cervical thymus
22 of veal calves. The thoracic thymus of veal calves treated with dexamethasone and estradiol
23 showed a decreasing of FKBP51 ($P < 0.05$), FKBP52 ($P < 0.05$), glucocorticoid receptor ($P <$
24 0.05) and MR expression ($P < 0.05$) compared to control group in the thoracic thymus of veal
25 calves. The gene expression of FKBP51 decreased both in cervical ($P < 0.01$) and thoracic
26 thymus ($P < 0.01$) of beef cattle treated with dexamethasone and estradiol. Additionally, also
27 prednisolone administration reduced FKBP51 expression in the cervical thymus ($P < 0.01$)
28 and in the thoracic thymus of beef cattle ($P < 0.01$). The gene expression of FKBP52
29 increased only in the cervical thymus following dexamethasone administration ($P < 0.01$). The
30 decrease of FKBP51 gene expression in thymus could be a possible biomarker of illicit
31 dexamethasone administration in bovine husbandry. Moreover, so far an effective biomarker
32 of prednisolone administration is not identified. In this context, the decrease of FKBP51 gene
33 expression in thymus of beef cattle following prednisolone administration could play an
34 important role in the indirect identification of animals illegally treated with prednisolone.

35

36 **Keywords:** thymus, bovine, FKBP51, FKBP52, glucocorticoids, real time PCR

37 **1. Introduction**

38 Despite the European Union ban on the use of the synthetic glucocorticoids (GCs) for growth-
39 promoting purposes in bovine livestock [1], these molecules are illegally administered, either
40 alone or in association with anabolic steroids, to improve quality and quantity of meat in veal
41 calves and beef production [2]. However, the analytical methods adopted by official
42 monitoring programs are unable to detect unknown molecules or different drugs administered
43 in combination at very low dosages [2]. So, novel approaches, such as the target organ
44 histology and “*omics*” techniques, have been proposed as screening tools to identify
45 secondary markers of illicit treatments, irrespective of the molecule used [3-7]. In this respect,
46 the thymus atrophy is considered an indirect biomarker of corticosteroid administration in
47 bovine [8-10] and the thymus histology is officially adopted by the Italian National Program for
48 Residue Surveillance (PNR).

49 The functions of GCs are mediated via their intracellular glucocorticoid (GR) and
50 mineralocorticoid receptor (MR). The affinity of the MR for GCs is high, so that it is
51 substantially occupied under basal conditions, whereas GR are activated with high plasma
52 corticosteroid levels. In the absence of ligand, corticosteroid receptors remain sequestered in
53 complex with chaperone and co-chaperone proteins including HSP90, HSP70, a 23-kDa co-
54 chaperone (p23) and FK506 binding protein 51 (FKBP51) or FK506 binding protein 52
55 (FKBP52). The association of FKBP52 with receptor-chaperone complexes results in an
56 enhancement of receptor hormone binding [11-13] and allows the nuclear translocation of the
57 complex [14]. In the nucleus the receptor complexes promote the gene transcription, including
58 FKBP51 gene, whose product competes with FKBP52 for the acceptor binding site on HSP90.
59 The receptor-enhanced expression of FKBP51 moves the equilibrium towards the FKBP51-
60 containing complexes which bind with lower affinity to GCs, resulting in impaired nuclear

61 translocation of the receptor [15]. Interestingly, FKBP51 but not FKBP52, is up-regulated by
62 steroid hormones [16-19], excluding estrogens [20], rendering the FKBP51 protein as a
63 component of an ultra-short regulatory loop in steroids signaling.

64 An important additional level of regulation is represented by 11 β -hydroxysteroid
65 dehydrogenases (11 β -HSDs). Indeed, 11 β -HSD1 regenerates the active form of GCs,
66 whereas 11 β -HSD2 metabolizes active GCs into inactive derivatives [21] thereby protecting
67 the MR from occupation by endogenous GCs [22].

68 The aim of this study was to investigate how a chronic exposure to synthetic GCs, such as
69 dexamethasone (DEX) and prednisolone (PRD), affects the molecular mechanisms
70 modulating the response to these hormones. So, the gene expression of GR, MR, 11 β -HSD1,
71 and 11- β HSD2 was evaluated in thymus of veal calves treated with DEX or PRD. Moreover,
72 the expression of FKBP51 and FKBP52 in the thymus of veal calves and beef cattle
73 experimentally treated with GCs was investigated to establish whether the FKBP51 gene may
74 be considered as a biomarker for the detection of GCs abuse in bovine husbandry.

75

76

77 **2. Materials and methods**

78 **2.1. Animals and experimental design**

79 The experiments were authorised through the Italian Ministry of Health and the Ethical
80 Committee of the University of Turin. The carcasses of the treated animals were appropriately
81 destroyed (2003/74/CE – DL 16 March 2006, No. 158).

82 In trial 1, twenty-two Friesian male veal calves at approximately 4 mo of age were used. The
83 calves were housed in 10 x 15 m boxes, with concrete floors lacking litter or lateral partitions.

84 The calves were tethered and fed with liquid milk replacer twice a day (providing per kg: 950 g

85 dry matter (DM), 230 g crude protein (CP), 210 g ether extract (EE), 60 g ash, 1 g cellulose,
86 75 mg retinol, 50 mg ascorbic acid, 5 mg Cu, 0.125 mg cholecalciferol, and 80 mg α -
87 tocopherol). The amount of feed was increased gradually to 8 L/calf/d and then gradually
88 increased to 16 L/calf/d. After one mo, 0.5 kg of barley straw (per kg: 900 g DM, 20 g CP, 10
89 g EE, 60 g ash, and 410 g crude fibre) was added to the diet, according to the
90 recommendations of the European Commission (97/182/EC). The calves were randomly
91 assigned to 4 experimental groups at approximately 5 mo-old: group A (n = 6) was weekly
92 administered 5 mg/animal of estradiol benzoate intramuscular for six wk in combination with
93 0.40 mg/animal/d of dexamethasone (DEX) *per os* for the last 31 d of treatment; group B (n =
94 8) was administered 15 mg/animal/d of prednisolone (PRD) *per os* for 31 d; group K1 (n = 8)
95 served as control. The calves were slaughtered at 3 d after the last treatment.

96 In trial 2, eighteen Charolaise male beef cattle (17 to 22 mo-old) were used. The animals
97 were housed in 10 x 15 m boxes with concrete floors lacking litter or lateral partitions. All
98 animals were fed a concentrated diet comprising corn silage, corn, hay, and a commercial
99 protein supplement; water was supplied *ad libitum*. The beef cattle were randomly assigned to
100 3 experimental groups: group C (n = 6) was administered with 0.70 mg/animal/d of DEX *per*
101 *os* for 40 d; group D (n = 6) was administered 15 mg/animal/d of PRD *per os* for 35 d; group
102 K2 (n = 6) served as control. The beef cattle were slaughtered at 6 d after the last treatment.
103 The animals resulted healthy upon intra-vitam and post-mortem examinations.

104

105 **2.2. Tissue sampling and processing**

106 Samples of cervical and thoracic thymus were obtained from each animal at slaughterhouse.
107 The samples were immediately frozen in liquid nitrogen or placed in 5 to 10 volumes of
108 RNAlater Solution (Ambion) and then stored at -80 °C for molecular analyses.

109

110 **2.3. RNA extraction, reverse transcription and qPCR**

111 Several milligrams of each tissue sample were disrupted using a TissueLyser II (Qiagen,
112 Hilden, Germany) using stainless steel beads in 1 mL of TRIzol Reagent (Ambion) according
113 to the manufacturer's protocol. The RNA concentration was spectrophotometrically
114 determined, and the RNA integrity was evaluated using an automated electrophoresis station
115 (Experion Instrument, Bio-Rad, Hercules, CA). Using the QuantiTect Reverse Transcription
116 Kit (Qiagen), cDNA was synthesised from 1 µg of total RNA.

117 The effect of treatments on FKBP51 and FKBP52 mRNA expression in the cervical and
118 thoracic thymus of veal calves and beef cattle was evaluated through quantitative polymerase
119 chain reaction (qPCR). The evaluation on GR, MR, 11β-HSD1 and 11β-HSD2 mRNA
120 expression was limited to veal calves (trial 1), because the expression of these genes in
121 thymus of beef cattle (trial 2) was previously reported [3]. To determine the relative amounts
122 of the specific transcripts, the cDNA obtained from retrotranscription was subjected to qPCR
123 [23] using the IQ5 detection system (Bio-Rad) and respective gene primers in an IQ SYBR
124 Green Supermix (Bio-Rad). Primer sequences of FKBP51 and FKBP52 were designed using
125 Primer3 (vers. 4.0.0) based on reference sequences NM_001192862 and NM_001034322,
126 respectively. Primer sequences of GR, MR, 11β-HSD1 and 11β-HSD2 were previously
127 reported [3]. The peptidylpropyl isomerase A (PPIA) gene was used as a housekeeping gene,
128 as previously described [3]. The expression level of each target gene was calculated using
129 the $2^{-\Delta Cq}$ method, where $\Delta Cq = Cq_{\text{target gene}} - Cq_{\text{housekeeping gene}}$ [24].

130

131 **2.4. Statistical analysis**

132 Statistical analyses were performed using Graph-Pad InStat (vers. 3.05) statistical software
133 (GraphPad Inc., San Diego, CA). The analysis of gene expression was performed using one-
134 way analysis of variance (ANOVA), followed by Dunnett's post-test. If Bartlett's test suggested
135 that the difference between the standard deviations of each group was significant, then the
136 nonparametric Kruskal-Wallis test with Dunn's post-test versus the control group was applied.
137 The Grubbs test was used to reveal potential outliers. A *P* value of < 0.05 was considered
138 statistically significant. The data are shown as the mean arbitrary units ($2^{-\Delta Cq}$) \pm SEM.

139

140

141 **3. Results**

142 In the cervical thymus of veal calves, the DEX administration in combination with estradiol
143 (group A) increased the expression of MR (mean of mRNA arbitrary units \pm SEM: 1.28×10^{-4}
144 $\pm 3.48 \times 10^{-5}$) compared with the control group K1 ($4.72 \times 10^{-5} \pm 8.34 \times 10^{-6}$) ($P < 0.01$) (Fig.
145 1B) and 11 β -HSD1 ($2.16 \times 10^{-4} \pm 1.88 \times 10^{-5}$) (Fig. 1C) compared with the control group K1
146 ($1.18 \times 10^{-4} \pm 2.17 \times 10^{-5}$) ($P < 0.05$). The same treatment also decreased FKBP51
147 expression ($7.75 \times 10^{-3} \pm 6.40 \times 10^{-4}$) compared with the control group K1 ($1.17 \times 10^{-2} \pm 6.84$
148 $\times 10^{-4}$) ($P < 0.01$) (Fig. 1E). No change of GR, 11 β -HSD2 and FKBP52 expression was
149 observed in the cervical thymus (Fig. 1A, D, F).

150 In the thoracic thymus the DEX administration in combination with estradiol (group A)
151 decreased GR expression ($2.58 \times 10^{-3} \pm 7.69 \times 10^{-4}$) ($P < 0.05$) compared with the control
152 group K1 ($5.74 \times 10^{-3} \pm 1.31 \times 10^{-3}$) (Fig. 1A), MR expression ($4.10 \times 10^{-5} \pm 4.04 \times 10^{-6}$)
153 compared with the control group K1 ($1.04 \times 10^{-4} \pm 2.19 \times 10^{-5}$) ($P < 0.05$) (Fig. 1B), FKBP51
154 expression ($5.40 \times 10^{-4} \pm 8.75 \times 10^{-5}$) compared with the control group K1 ($1.87 \times 10^{-3} \pm 6.92$

155 $\times 10^{-4}$) ($P < 0.05$) (Fig. 1E) and FKBP52 expression ($1.65 \times 10^{-3} \pm 4.08 \times 10^{-4}$) compared with
156 the control group K1 ($4.54 \times 10^{-3} \pm 1.24 \times 10^{-3}$) ($P < 0.05$) (Fig. 1F). No change of 11 β -HSD1
157 and 11 β -HSD2 expression was observed in the thoracic thymus (Fig. 1C, D).
158 The administration of DEX (group C) in beef cattle reduced FKBP51 expression in the cervical
159 thymus ($1.44 \times 10^{-2} \pm 1.87 \times 10^{-3}$) compared with the control group K2 ($8.08 \times 10^{-2} \pm \times 10^{-2}$) (P
160 < 0.01) (Fig. 2A) and in the thoracic thymus ($1.37 \times 10^{-2} \pm 2.42 \times 10^{-3}$) compared with the
161 control group K2 ($5.18 \times 10^{-2} \pm 1.02 \times 10^{-2}$) ($P < 0.01$) (Fig. 2A). Moreover, also PRD
162 administration (group D) reduced FKBP51 expression in the cervical thymus ($2.31 \times 10^{-2} \pm$
163 3.03×10^{-3}) ($P < 0.01$) (Fig. 2A) and in the thoracic thymus ($1.54 \times 10^{-2} \pm 4.19 \times 10^{-3}$)
164 compared with the control group K2 ($P < 0.01$) (Fig. 2A). The expression of FKBP52 gene
165 increased following DEX administration only in cervical thymus ($2.97 \times 10^{-2} \pm 3.33 \times 10^{-3}$)
166 compared to control group K2 ($1.73 \times 10^{-2} \pm 3.56 \times 10^{-3}$) ($P < 0.05$) (Fig. 2B).

167

168

169 **4. Discussion**

170 The administration of DEX caused a decrease of GR expression only in thoracic thymus of
171 veal calves, whereas no change was detected in cervical thymus. These findings are partially
172 consistent with data previously reported in thymus of beef cattle where GR expression did not
173 change in response to DEX treatment [3].

174 Although many studies showed that FKBP51 expression can be induced via GCs treatment
175 both *in vitro* [17,19] and *in vivo* [16,18], a decrease of FKBP51 expression in thymus of veal
176 calves and beef cattle following DEX administration was detected. Similar results in bovine
177 skeletal muscle following long term administration of DEX were reported [5].

178 Most of the information about FKBP51 is closely related to human and mouse, and very little
179 information is available about this gene in other animals. Moreover, it should be emphasised
180 that illicit schedules in veal calves and cattle substantially differ, in terms of dosage and
181 duration of administration, from those used in human. Furthermore, unlike the *in vitro*
182 experiments, GCs treatment was halted 3 or 6 d (trial 1 and 2, respectively) before the
183 slaughter, when the organs were sampled for molecular analysis. During this period, it is
184 conceivable that a restoration of the physiological conditions occurs. Indeed, Scharf and
185 colleagues [18] observed that FKBP51 mRNA was significantly up-regulated 4 h and 8 h after
186 a single DEX treatment in the central amygdale of mouse, but returned to baseline already
187 after 24 h. Additionally, even GR down-regulation induced by GCs over-stimulation was
188 reversed by DEX withdrawal [25].

189 Nevertheless, the subcellular localization of steroid receptors and thereby their activity is also
190 affected by other factors, such post-translational modifications, redox milieu or protrusion of
191 localization signals [reviewed in 26,27].

192 The long term administration of GCs has been related to many adverse systemic effects. In
193 particular, the hypothalamic-pituitary-adrenal (HPA) axis is suppressed and the full recovery
194 of the suppressed adrenal response may take more several months after the GCs withdrawal
195 [28]. However, the degree of suppression can be affected by several factors, such as the
196 duration of treatment, type of steroid employed and dosage, as well as by the route of the
197 drug administration [reviewed in 29]. Then, it reasonable to suggest that a prolonged
198 exposure to GCs leads to the suppression of the HPA axis also in calves and beef. The
199 consequently reduction of serum cortisol influences the GR expression with a finally decrease
200 of FKBP51 gene expression. Moreover, there is increasing evidence that epigenetic changes
201 in non-coding regions of FKBP51 gene may influence basal and hormone-stimulated

202 expression. Many studies reported that GCs decreased the FKBP51 DNA methylation [16,30]
203 and this reduction persisted for 1 to 4 wk after the GCs administration was discontinued [16].
204 However, experimental data suggest that the epigenetic patterns observed may be tissue-
205 specific [16,30] and probably other epigenetic mechanisms, like chromatin modifications,
206 would be implicated in the FKBP51 gene expression [31].

207 PRD treatment caused a decrease of FKBP51 expression only in the thymus of beef cattle,
208 but not of veal calves. Differences of gene expression in thymus of beef cattle between DEX
209 and PRD treatment have been reported [3]. The difference is not limited to changes of the
210 regulation of individual genes alone, but is also evident at morphological and histological level.
211 Indeed, the DEX treatment induces atrophy in the thymus and the parenchyma is replaced by
212 fat tissue [8,9], whereas no histological lesions were observed in the thymus of the PRD-
213 treated beef cattle [10].

214 These findings considered together suggest a differential response to DEX and PRD in veal
215 calves and beef cattle, perhaps because of differences in pharmacokinetics and/or
216 pharmacodynamics. Dexamethasone has a stronger anti-inflammatory action and a lower
217 mineralocorticoid effect than PRD [32]. Moreover, the biological half-life of PRD is minor
218 compared to DEX (<http://toxnet.nlm.nih.gov>). Because PRD has a weaker effect than DEX,
219 the biological effects of these synthetic GCs probably differ in duration.

220 The increase of 11 β -HSD1 and MR gene expression in the thymus of DEX-treated animals is
221 probably due to lymphatic tissue substitution by adipocytes. Indeed, adipocytes express
222 physiologically 11 β -HSD1 and MR genes to a greater extent than lymphocytes [33]. On the
223 contrary, in thoracic thymus the MR expression decreased, whereas no change of 11 β -HSD1
224 was detected. The differences between the portions of thymus may be due to different rate of
225 regeneration. Indeed, thymus preserves an intrinsic ability to regenerate after GCs

226 administration because the bovine thymic parenchyma and activity could be restored, as
227 previously shown by gross and histological observations [9].

228 Although many articles reported that the expression of FKBP52 gene is not under steroids
229 control, except estrogen [20,34], FKBP52 gene expression changed in thoracic thymus of
230 veal calves and in cervical thymus of beef cattle following DEX treatment. It is possible that
231 the prolonged treatment with DEX finally caused a change in FKBP52 gene expression.
232 Moreover, DEX administration could directly cause no change of gene expression, but
233 through the activation of different indirect mechanisms of regulation.

234 Regarding estrogens, most studies about FKBP52 are related to *in vitro* experiments using
235 tumor cells [20,34] and very little information is available about this gene *in vivo*. However, the
236 molecular interactions that mediate constitutive or regulated gene activity of this gene are
237 largely unexplored [35] and further studies are needed to better evaluate the mechanism
238 regulating FKBP52 gene expression.

239 In conclusion, the results of our experiments demonstrated that GCs specifically induce a
240 decrease of the FKBP51 mRNA levels in the thymus of veal calves and beef cattle. This
241 finding could allow the application of FKBP51 expression as a indirect biomarker in a
242 screening test to identify the animals illegally treated with GCs in bovine husbandry. Above all,
243 the gene expression decrease induced by PRD in thymus of beef cattle appears of particular
244 interest because so far an effective biomarker of PRD administration is not identified.

245 Therefore, the implications of these results have greater relevance for their potential
246 application to food safety monitoring, considering that changes of the FKBP51 gene
247 expression may persist for up to a week after the suspension of GC treatment.

248

249

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254 The authors declare no conflicts of interest.

255

256

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391 **Figure captions**

392 **Fig. 1.** Effects of dexamethasone in combination with estradiol benzoate (group A) or
393 prednisolone (group B) on GR (A), MR (B), 11 β -HSD1 (C), 11 β -HSD2 (D), FKBP51 (E) and
394 FKBP52 (F) gene expression compared with the control group K1 in the cervical and thoracic
395 thymus of veal calves. The results are presented as the means \pm SEM. The y-axes show
396 arbitrary units representing relative mRNA expression levels. * P < 0.05, ** P < 0.01 versus the
397 control group K1.

398

399 **Fig. 2.** Effects of dexamethasone (group C) or prednisolone (group D) on FKBP51 (A) and
400 FKBP52 (B) gene expression compared with the control group K2 in the cervical and thoracic
401 thymus of beef cattle. The results are presented as the means \pm SEM. The y-axes show
402 arbitrary units representing relative mRNA expression levels. * P < 0.05, ** P < 0.01 versus the
403 control group K2.