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Action of larch bark in the regulation of cortisol induced stress in sheep

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ABSTRACT - The effect of Larix decidua (LD) dietary administration on gene expression patterns has been evaluated in sheep under ACTH challenge. Experimental protocol was approved by local laws and regulations. Eighteen sheep at maintenance were allotted to 3 groups: CTR (negative control, without ACTH, and supplementation), ACTH (positive control, with ACTH, and without supplementation), LD (ACTH and 50 g/head/day of LD). ACTH was injected for 3 subsequent days to ACTH and LD groups and blood was sampled before (T0) and after 3 (T3) and 51 (T51) hours from the first injection. RNA extracted samples were pooled together within group and time of sampling. A custom oligoarray was synthesized using 24,384 35-40mer probes designed from 12,194 UniGenes (NCBI) on a CombiMatrix 90K platform. Cy5 labelled samples were hybridized on the chip. Statistical analysis, performed with MeV software 4.1 (TIGR), allowed the identification of a set of genes which were up or down regulated as a consequence of ACTH treatment. Genes that resulted differentially expressed were annotated with HomoloGene system and data mining was performed with Babelomics v3.1 tool. Functional analysis showed that most of the differentially expressed genes belong to KEGG pathways involved in immune system response and signaling molecules and interaction. Larch administration was effective in counteracting the effect of ACTH injection on the inflammatory processes, restoring the physiological homeostasis.

Key words: Nutrigenomics, Microarray, Stress, Sheep.

Introduction – The role of bioactive compounds in enhancing the endogenous defences, through the regulation of gene expression, is a milestone in nutrigenomics studies on humans and laboratory animal models (Barnes, 2008; Konkimalla and Efferth, 2008). For this reason, the use of plant remedies, known to possess natural antioxidant, immunomodulatory and antiinflammatory properties, has increased in the last decade in humans. The interest for these bioactive compounds has been extended also to the livestock production system (Stefanon et al., 2005; Sgorlon et al., 2006, 2007; Colitti et al., 2007), since the increasing levels of production of high genetic merit animals and the use of technologies to enhance the productions cause frequently stress-related syndromes and an impairment of the immune function (Goff and Horst, 1997). Recent studies demonstrated the beneficial role of plant compounds in regulating stress and immune response also in livestock production species. In a recent study, Echinacea angustifolia and Andrographis panicolata showed regulatory effects on inflammatory conditions in sheep (Sgorlon et al., 2008). Moreover, the residues of several plant extracts resulted able to modulate immuno functions of sheep neutrophils and, in the specific case of Larix decidua (larch, LD), it was also evident a strong anti-inflammatory activity (Farinacci et al., 2008).

Starting from these encouraging results, the trial aimed at investigating the regulatory effects of Larix decidua on the blood transcriptome of sheep submitted to ACTH challenge.
Material and methods – Eighteen Sarda sheep, homogeneous for age and body conditions, were randomly assigned to three groups (CTR, ACTH, and LD) and fed twice a day a basal diet (1 kg/head day of unipellet) formulated to cover maintenance requirements. In addition to the basal diet, sheep of LD group received 0 g/head/day of Larix decidua sawdust administered at the morning meal. After 22 days of adaptation, the sheep of ACTH and LD groups were injected twice a day with 0.05 mg of ACTH (Synachten, Novartis) for a period of 3 days, whilst the sheep of CTR group received the same dose of saline. Blood samples were collected from jugular vein in the morning, before (T0) and after 3 (T3) and 51 (T51) hours from the first ACTH injection. The extraction of mRNA from blood samples was performed with PAXgene blood RNA kit (PreAnalitiX, Qiagen). Absorbance measurements at 260 nm and 280 nm were used to assess the yield of extraction and the purity of RNA samples. Integrity of RNA was assessed by mean of Agilent 2100 Bioanalyzer. Samples were then pooled together within group and time of sampling, including in the pool an equal quantity of mRNA for each sheep. Nine pools were obtained for the hybridizations. Each pool was submitted to alpha and beta globin mRNA depletion adapting human GLOBINclear commercial kit (Ambion, Inc.) to sheep. Samples were again quantified by spectrophotometer and 100 ng of each pool were submitted to double round of amplification using Amino Allyl MessageAmp™ II aRNA Amplification Kit (Ambion, Inc.). At the end of amplification protocol, aRNA was labeled with Cy5 dye and hybridizations were performed following the instructions supplied by CombiMatrix with CustomArray 90K. The oligoarray was synthesized using 24,384 35-40mer probes designed from 12,194 UniGenes (NCBI - Build #13) by mean of OligoWiz 2.0 software. The microarray was scanned using a Perkin Elmer laser scanner and data were extracted from the image using CombiMatrix Microarray Imager Software.

Raw data were normalized using the function “Normalize Gene/Row Vectors” of MeV software v 4.1 (TIGR) and were analyzed with a two-way (treatment and time) ANOVA (MeV software v 4.1). Results were considered statistically significant for p-values<0.001 and differentially expressed genes were submitted to hierarchical clustering (MeV software v.4.1 – TIGR). Genes were annotated with HomoloGene system and data mining was performed with FatiGO tool (Babelomics v3.1).

Results and conclusions – Two-way ANOVA allowed identifying 266, 111, and 181 genes differentially expressed for treatment, time and the interaction, respectively. Hierarchical clustering (HCL) of differentially expressed genes was used to generate heatmaps for each of these effects. HCL tree generated for genes differentially expressed for treatment effect (Figure 1) showed that ACTH group at T3 and T51 belongs to a cluster different from that including CTR and LD groups. These results underline the effects of ACTH challenge on gene expression patterns as observed in previous studies in sheep (Sgorlon et al., 2008). Moreover, CTR sample at T51 and T0 and LD sample at T0, T3, and T51 were grouped to a unique cluster, indicating that larch administration counteracted the effects induced by ACTH injection. HCL for interaction strengthen this hypothesis, since the groups CTR at T0, ACTH at T0, LD at T3 and LD at T51 revealed the same trend of expression. Functional analysis showed that major number of differentially expressed genes are involved in KEGG pathways “Toll-like receptor signaling pathway”, “T cell receptor signaling pathway”, “Neuroactive ligand-receptor interaction”, “Jak-STAT signaling pathway”, and “Cytokine-cytokine receptor interaction”. From these results, we can consider that larch administration is effective in modulate immune system response not only in vitro (Farinacci et al., 2008) but also in vivo, through the regulation of gene expression patterns. A preliminary data mining showed that large part of these genes belongs to biological processes related to “cell communication”, “cell differentiation”, “response to stress”, “cell death” and “apoptosis”.

In conclusion, the trial pointed out the biomolecular mechanisms involved in response to ACTH challenge and demonstrated the ability of Larix decidua in counterbalance these effects.
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