**Phenotypic characterization of encephalitis in the brains of goats experimentally infected with Spanish Goat Encephalitis Virus**

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**ABSTRACT**

Spanish goat encephalitis virus (SGEV) is a novel tick-borne flavivirus subtype, closely related to the flavivirus louping ill virus (LIV). SGEV caused a severe, acute and mortal neurological disease outbreak in northern Spain in a goat herd. In order to characterize the cell population in lesions and to determine the distribution of the inflammatory cells, central nervous system (CNS) samples of nine female Alpine goats challenged subcutaneously with SGEV over the right thorax behind the elbow were evaluated using immunohistochemistry (microglia-Iba1, T lymphocytes-CD3, B lymphocytes-CD20 and astrocytes-GFAP). The number of microglia (37.8%) and T lymphocytes (21.5%) was greater than the number of B lymphocytes (16.8%). Goats were classified into clusters based on the severity of histological lesions in CNS (A-mild to moderate lesions and B-severe lesions). Microglia was significantly more abundant than T and B lymphocytes in cluster B (severe lesions). The total area occupied by glial foci revealed that medulla oblongata and spinal cord were the most affected tissues. Astrogliosis (GFAP+) was present in the majority of the CNS sections being greater in the pial surface. The lesion predominance on the right side of the medulla oblongata, which could be associated to the site of challenge suggestive of neurotropic route was also statistically confirmed. Results suggest that the cellular immune response would be the most important response to the SGEV infection.

**Keywords:** Spanish Goat Encephalitis Virus, goat, cell population, immunohistochemistry

**1. Introduction**

Human and animal shared infectious diseases such as vector-borne diseases have been a permanent threat to human health through the centuries causing social and economic damage related to unexpected illnesses and deaths (Howard and Fletcher, 2012; Morens and Fauci, 2013). Tick-borne viruses are examples of those viruses continually spreading to new geographic locations, influenced by human encroachment and environmental conditions such as agricultural and livestock activities, urbanization or global trade (Howard and Fletcher, 2012; Marston et al., 2014; Mansfield et al., 2017). Climate change has increased temperature, therefore vectors may extend to previously unaffected areas (Mansfield et al., 2009), helped by migrating birds or other susceptible wildlife hosts (Mansfield et al., 2009; 2017). The flavivirus louping ill virus (LIV) was one of the first tick-borne viruses identified (Kazimírová et al., 2017; Mansfield et al., 2017). LIV would appear to have evolved from an ancestral tick borne encephalitis (TBE)-like virus (TBEV) some 400 years ago (Reid, 1999). Louping ill is an endemic disease of the British Isles and Ireland caused by LIV and its distribution is associated with the presence of its main vector, the hard tick *Ixodes ricinus* (Jeffries et al., 2014; Buxton and Reid, 2017). LIV was initially isolated from sheep (Smith et al.,1964a), red grouse (Williams et al.,1963) and cattle (Twomey et al., 2001), but it has also been reported in other animal species, including goats (Gray et al.,1988), horses (Fletcher, 1937), dogs (MacKenzie et al.,1973), pigs (Bannatyne et al.,1980), deer (Reid et al., 1976), mountain hares (Smith et. al.,1964b), alpacas (Cranwell et.al, 2008) and llamas (Nettleto et al., 2014). LIV causes a febrile illness and acute encephalomyelitis (Jeffries et al., 2014). In September 2011 a severe and acute neurological disease outbreak occurred in northern Spain in a goat herd (Balseiro et al., 2012) and the isolated virus was identified and characterized as a novel tick-borne flavivirus subtype, closely related to LIV. It was designated Spanish goat encephalitis virus (SGEV) (Mansfield et al., 2015).

The pathogenesis of flavivirus in the central nervious system (CNS) involves complex virus–host interactions. In acute viral encephalitis, the recruitment of immune cells into the CNS plays a fundamental role in the outcome of the disease (Maximova et al., 2009). Considerable progress has been made in recent years in understanding host defenses to flavivirus infection (Dörrbecker et al., 2010). In contrast to the humoral immune response, the cell-mediated immune response elicited against natural infection has been rather poorly studied until recently (Blom et al., 2018). Although not specifically explored in current studies, both humoral and cell-mediated immune responses are clearly important factors in determining the outcome of flavivirus infections (Gelpi et al., 2005). An intrinsic CNS cell response consisting of microglia, the principal immune effector and the first cell type to respond and, astrocytes which undergo changes in its morphology, with marked increase in the expression of glial fibrillary acidic protein referred to as reactive astrogliosis has been observed (Rock et al., 2004; Palus et al., 2014; Maximova and Pletnev, 2018). Indeed astrocytes might be a potential source of proinflammatory cytokines and thus contribute to the neurotoxicity and blood-brain barrier breakdown and consequently to the multiplication of flaviviruses in the brain (Palus et al., 2014; Potokar et al., 2019). The extrinsic response on the other hand is predominantly based on T lymphocytes with B lymphocytes rarely found, as has been observed in other viral encephalitis (Maximova et al., 2009; Hatanpaa and Kim, 2014). Cell-mediated immune reactions in the CNS may contribute to neural damage with severe consequences of brain function, and could in the worst cases lead to a fatal outcome (Gelpi et al., 2005). Characterization of cell populations has been studied in humans and in non-human primates infected with the TBEV (Gelpi et al., 2005; 2006; Růžek et al., 2009), in mice and lambs experimental infected with LIV (Sheahan et al., 2002), in horses infected with the West Nile virus (Delcambre et al., 2017), and in humans and non-human primates infected with others flavivirus (Maxivoba et. al., 2009; Maxinova and Pletnev, 2018). Knowledge gained in the field of the immunopathogenesis of diseases affecting the CNS could be helpful in understanding flavivirus disease patterns (Blom et al., 2018).

The purposes of this study were (i) to describe the inflammatory response within the CNS of goats challenged subcutaneously with SGEV and, (ii) to determine the phenotype and distribution of inflammatory cell, astrocytes and microglial cells.

**2. Materials and methods**

*2.1. Animals and sampling*

Nine female Alpine goats, identified as 76, 77, 78, 79, 80, 81, 82, 85 and 86, were challenged subcutaneously with SGEV, over the right thorax behind the elbow (Salinas et al., 2017). They were sacrificed at 12, 21 and 28 days post challenge by intravenous overdose of pentobarbital (Salinas et al., 2017). CNS samples consisted of sections of cerebral cortical regions, corpus callosum, thalamus, hypothalamus, hippocampus, midbrain, cerebellum, pons, medulla oblongata and four sections of the spinal cord (cervical, thoracic, lumbar and sacral) were taken for histopathology. Microscopic lesions found on those animals were ascribed to one of three levels of increasing severity using haematoxylin and eosin staining (Salinas et al., 2017): grade I, only perivascular cuffing; grade II, perivascular cuffing and small foci of glial cells; and grade III, nonsuppurative encephalomyelitis. The latter consisted of perivascular cuffing formed by infiltrates of lymphocytes and histiocytes, diffuse or focal proliferation of glial cells, neuronal degeneration, neuron necrosis and neuronophagia, demyelination and vacuolation of the neuropil and meningitis, and microvascular changes consisting of reactive endothelium and perivascular oedema. Histological lesions were consistently more severe in the midbrain, cerebellum, medulla oblongata and cervical spinal cord (Salinas et al., 2017). Immunohistochemical (IHC) examination for detection of SGEV was also performed in those samples although no extensive positive labelling for SGEV was found by IHC in any brain section from any animal in the study (Salinas et al., 2017).

*2.2. Immunohistochemistry (IHC)*

For each goat, serial paraffin-embedded sections (4 µm) were used for immunohistochemical detection of four cell populations using different primary antibodies: ionized calcium binding adaptor molecule 1 (Iba1) for microglial cells, CD3 for T lymphocytes, CD20 for B lymphocytes and glial fibrillary acidic protein (GFAP) for astrocytes. After being deparaffinised, the endogenous peroxidase activity was blocked by incubating sections with 0.5 % H2O2 in distilled water for 30 min. Then, epitope unmasking techniques (Table 1) were used to retrieve the antigens and samples were treated to prevent unspecific binding with a 20 min incubation in a humidified chamber with 5% normal serum and 0.1% bovine serum albumin (BSA) in tris buffered saline (TBS) 1x. The tissue sections were incubated overnight at 4°C in a humidified chamber with commercial monoclonal and polyclonal antibodies diluted in TBS+BSA 0.1% (Table 1), slides were washed with TBS 1x, incubated with a secondary antibody (Vector Laboratories, California, USA), diluted 1:200 in TBS+BSA 0.1% followed by incubation with the Avidin-biotin-peroxidase complex reagent-method (ABC Standard, Vector Laboratories, California, USA) in TBS 1x for 30 min. Labeling was visualized with application of 3,3'-diaminobenzidine tetrahydrochloride (DAB, Vector Laboratories, California, USA) as chromogen substrate. Slides were counterstained with Mayer´s haematoxylin, dehydrated and mounted with DPX (Fluka, Sigma, St. Louis, MO, USA). Negative control consisted of an additional slide with absence of the primary antibody. Lymph node tissue was used as a positive control for Iba1, CD3 and CD20 antibodies. Samples of CNS of a health goat were used as positive control for GFAP antibody.

*2.3. Evaluation and quantification*

Stained slides were studied under light microscopy Nikon, Eclipse E600® (Nikon, Japan) with digital light camera Nikon, DS-Fi1® (Nikon, Japan) and cells were count by imaging software Nikon, NIS-Elements BR® (Nikon, Japan). For cell population types positive staining was evaluated by obtaining 5 random parenchymal fields at 200x (Delcambre et al., 2017). For the analysis of microglia, T lymphocytes and B lymphocytes, we used quantitative assessment, counting the immunolabeled cells of the total number of cell within the perivascular cuffing and glial foci. Astrocytes were evaluated with a semi- quantitative scoring system adapted from previous study on reactive astrogliosis in humans disorders (Sofroniew and Vinters, 2010): 1; astrocytes in healthy CNS tissue (not all astrocytes express detectable levels of GFAP); 2; mild to moderate reactive astrogliosis; 3; severe diffuse reactive astrogliosis; score 4; severe reactive astrogliosis with compact glial scar formation.

In order to estimate the total area occupied by all glial foci, the percentage of the affected area respect to the total area of the CNS section was calculated (the glial foci were intentionally searched and its area was measured). For that purpose we employed the software image-processing program Image J®. Additionally to evaluate the spatial distribution (laterality) of the glial foci, medulla oblongata sections were scanned by light microscopy Olympus, BX51® (Olympus, Japan) and the image analyzed by software Olympus, VS-ASW 2.8® (Olympus, Japan). We selected medulla oblongata because that tissue was consistently affected and had a similar size in all studied goats (Salinas et al., 2017).

*2.4. Statistical analysis*

Proportion (average of the percentages) of immunolabelled cell counts (microglia, T and B lymphocytes) was submitted to an arc sinus square root transformation in order to meet the normality criterion for statistical analyses. Then in a first approach they were submitted to a cluster analysis by the method of the centroid in the SAS CLUSTER procedure (SAS Institute Inc., Cary, NC, USA) (Fig. 1). Two clusters were clearly defined. Goats 78, 79, 80 and 82 fell into cluster A and goats 76, 77, 81, 85 and 86 into cluster B (Salinas et al., 2017). These two clusters were considered as overall microscopic lesions of three levels; grade I and II (cluster A-moderate lesions) and III (cluster B-severe lesions) (Salinas et al., 2017) and therefore introduced into the dataset as a new variable (grade) in order to represent population variability in a more meaningful way than just individual values. Then microglia (Iba1), T lymphocytes (CD3) and B lymphocytes (CD20) transformed cell count proportions, as well as astrocytes (GFAP) log transformed scores and affected Iba1 stained area proportion were used as dependent variables in a General Linear Model (GLM) analysis with the SAS GLM procedure (SAS Institute Inc., Cary, NC, USA) according to region, type of lesion (perivascular cuffing or glial foci) and cluster and their first order interactions. Pairwise least square means comparisons were carried out with the Student’s t test from the LSMEANS statement in the SAS statistical package (SAS Institute Inc., Cary, NC, USA). Statistical significance was accepted at the *P* < 0.05, and tendency at the *P* = 0.05 - 0.1.

Laterality effect in the number of lesions (glial foci) was tested with the Chi square test in the FREQ procedure from the SAS statistical package version (SAS Institute Inc., Cary, NC, USA) and further confirmed on the log transformed foci counts submitted to the GLM procedure analysis of variance and lest square means Student’s t test with the LSMEANS statement in a model with two independent variables: cluster and side and their interaction.

*2.5 Ethical issue*

Sampling procedures and SGEV challenge were approved by the Animal Research Ethics Committee of the Community of Junta de Castilla y León, Spain (reference number ULE\_010\_2015). Experiments were conducted in accordance with the current Spanish and European legal requirements and guidelines regarding experimentation and animal welfare, from a previous study by Salinas et al. (2017).

**3. Results**

*3.1. Iba1 (Microglia)*

Microglia was the most abundant cell type accounting for an average of 37.8% of cells. Severity of the lesions determined statistical differences between clusters, microglia being statistically more abundant in cluster B-severe lesions (53.1%) than in cluster A-moderate lesions (22.4%; *P* < 0.0001) (Table 2, Figs. 2a and 2b). A statistically higher proportion of microglia were found in thalamus, hypothalamus and corpus callosum (53.1%) and in medulla oblongata (49.6%; *P* = 0.0065) than in cervical (29.8%; *P* = 0.0264) and lumbosacral spinal cord (20.9%; *P* = 0.0023). No differences were observed between microglia count into perivascular cuffing (37.2%) and glial foci (38.4%) in any regions (*P* = 0.9266).

Regarding the total area occupied by all glial foci, cluster B-severe lesions (14.6%) had significantly larger proportions of affected areas than cluster A-moderate lesions (2.2%; *P* = 0.0007). The regions with significantly larger proportion of affected areas were cervical (16.9%; *P* = 0.0003), lumbosacral spinal cord (13.4%; *P* < 0.0001) and medulla oblongata (12.6%; *P* = 0.0094). Pons and cerebellum (4.8%; *P* = 0.0214) also had significantly larger proportion of affected areas than hippocampus (3.60%; *P* = 0.0022) and the cortical area (1.7%; *P* = 0.0021).

*3.2. CD3 (T lymphocytes)*

T lymphocytes were the second most abundant cell type (21.5%) and, like microglia, were observed in a higher proportion in cluster B-severe lesions (26.5%) than in cluster A-moderate lesions (16.4%; *P* = 0.0001) (Table 2, Figs. 2c and 2d). T lymphocytes reached higher proportions in thalamus, hypothalamus and corpus callosum (32.6%) than in lumbosacral spinal cord (9.9%; *P* = 0.0008). Scant differences were observed between cell counts in perivascular cuffing (18.0%) and glial foci (24.9%; *P* = 0.2608).

*3.3. CD20 (B lymphocytes)*

B lymphocytes were the least abundant cell type (16.8%) and were also present in a higher proportion in cluster B-severe lesions (22.8%) than in cluster A-moderate lesions (10.8%; *P* < 0.0001) (Table 2, Figs. 2e and 2f). All regions seemed to have similar proportions of CD20 immunolabelled cells (*P* = 0.4820) with these ranging from 23.1% in pons and cerebellum to 11% in lumbosacral spinal cord. Scant differences were observed between cell counts in perivascular cuffing (19.3%) and glial foci (14.3%; *P* = 0.2166).

*3.4. Proportion of microglia (Iba1), T lymphocytes (CD3) and B lymphocytes (CD20)*

The proportion of microglia in all regions of the CNS examined was always higher than T and B lymphocytes (Table 2). This difference was only statistically significant in the thalamus, hypothalamus and corpus callosum (Iba1 vs CD20; *P* = 0.0028), hippocampus (Iba1 vs CD3; *P* = 0.0451, Iba1 vs CD20; *P* = 0.0238), midbrain (Iba1 vs CD3; *P* = 0.0194); Iba1 vs CD20; *P* = 0.0020) and medulla oblongata (Iba1 vs CD3; *P* = 0.0033). A tendency was observed in thalamus, hypothalamus and corpus callosum between Iba1 vs CD3 (*P* = 0.0696) and medulla oblongata between Iba1 vs CD20 (*P* = 0.0873). There were no statistically significant differences between the proportion of T and B lymphocytes in any regions.

When the analysis was carried out taking into account the intensity of the lesions, it was found that the proportion of microglia was statistically higher than T lymphocytes (*P* <0.0001) and B lymphocytes (*P* <0.0001) in the severe lesions (cluster B) while in the mild to moderate lesions (cluster A) significance was only detected between the microglia and the B lymphocytes (*P* = 0.0261). No differences were observed in the proportion of T and B lymphocytes conditioned by the intensity of the lesion.

*3.5. GFAP (Astrocytes)*

Cells immunolabeled with GFAP were ascribed to the following score: 1 (Fig. 3a), 2 (Fig. 3b) and 3 (Fig. 3c). Score 4 was not observed in any section of the CNS. Astrocyte frequency score differences were only observed between regions (*P* < 0.0001), but not between clusters A-moderate lesions (1.7%) and B-severe lesions (1.8%; *P* = 0.6997) (Table 2), nor perivascular cuffing and glial foci (1.8% vs 1.8%; *P* = 1.0000). Thalamus, hypothalamus, corpus callosum and lumbosacral spinal cord had the highest mean score (2.2%). Pons and cerebellum were the regions with the lowest score (1.3%) (Table 2).

*3.6. Lesion count and laterality*

Lesions were more frequent on the right side (73.7%) than on the left side (26.3%; *P* = 0.0035) (Fig. 4). The total mean lesion count statistically differed between the right side (2.9) and the left (1.0; *P* = 0.0239), and between cluster B-severe lesions (3.3) and cluster A-moderate lesions (0.6; *P* = 0.0034).

**4. Discussion**

This study describes, for the first time, the cell population and the distribution of lesions in CNS from goats experimentally infected with SGEV. The inflammatory cells infiltrating the perivascular space and the parenchyma of the CNS were predominantly microglia, with a moderate number of T lymphocytes and B lymphocytes. These findings are similar to those found in studies on LIV and others flaviviruses in horses, humans and non-human primates (Johnson et al., 1985; Sheahan et al., 2002; Gelpi et al., 2005; Maximova et al., 2009; Delcambre et al., 2017; Maxinova and Pletnev, 2018). It has been observed that B lymphocytes remain in the perivascular area (perivascular cuffing), whereas T lymphocytes move into the parenchyma (glial foci) in order to clear infected cells, i.e. the Japanese encephalitis virus and the tick-borne encephalitis virus (Johnson et al., 1985; Gelpi et al., 2006). The predominance of microglia in perivascular cuffing and glial foci in our study has been reported in mice, lambs, horses, humans and non-human primates with acute flavivirus encephalitis (Sheahan et al., 2002; Maxinova et al., 2009; Delcambre et al., 2017; Maximova and Pletnev, 2018). Microglia is the first cell type that responds to CNS infection, they are phagocytic cells quickly and unspecifically attracted by the first local cellular changes. The immune functions of microglia are regulated by cytokines, including interferon gamma (IFN-γ), the major mediator of macrophage activation. The activation of microglia results in signals for T lymphocytes infiltration and antigen presentation that probably contribute to the defense against invading pathogens (Rock et al., 2005).

T lymphocytes are the major population of lymphocytes found in mice, lambs, humans, non-human primates, horses and mice in flavivirus encephalitis studies (Sheahan et al., 2002; Gelpi et al., 2005; Gelpi et al., 2006; Maximova et al., 2009; Delcambre et al., 2017; Blom et al., 2018), with higher prevalences of CD8+ T than CD4+ T lymphocytes (Blom et al., 2018; Maximova and Pletnev, 2018 ). T lymphocytes play a key role and provide critical functions during the control of CNS viral infections by destroying virus infected cells, producing cytokines, increasing phagocytic activity of microglia, and stimulating the local production of antibodies by B lymphocytes (Binder, 2001).

In some studies B lymphocytes were rarely found (Johnson et al., 1985). Here numbers of B lymphocytes were slightly retained within the perivascular spaces (19.3%) compared to the parenchymal compartment (14.3%), similar to that observed in humans and non-human primates infected with flavivirus (Maximova et al. 2009; Maximova and Pletnev, 2018). Based on the data obtained, T lymphocytes were more numerous than B lymphocytes, although there were no significant statistical differences, probably associated to the smaller size sample. This finding would suggest that all goats were in an acute to subacute stage of illness, and the number of B lymphocytes would likely increase in the final stages of disease establishing a humoral response. More studies are needed to confirm this hypothesis.

The expression of GFAP in all CNS sections matches with what has been observed in LIV in mice and lambs (Sheahan et al., 2002), also in others flavivirus infections in humans and mice (Palaus et al., 2014; Potokar et al., 2019). The major presence of GFAP cells in thalamus, hypothalamus, corpus callosum and spinal cord compared to others regions may support the concept that astrocyte distribution and expression of GFAP is heterogeneous in regions of healthy CNS (Oberheim et. al., 2012). The majority of the CNS sections examined showed a score of 2 for astrogliosis characteristic of diffuse innate immune activation for viral infections (Sofroniew and Vinters, 2010). The score 3 for astrogliosis was observed near to the pial surface, likely because it is the first route of entry for the virus into the CNS. That means that the astrocytes are among the first cell types to come in contact with or intercept the virus. Similar result were reported in horses infected with WNV where the increase of expression of GFAP was not significant (Delcambre et al., 2017), suggesting that reactive astrogliosis is large in chronic or persistent infections (Potokar et.al., 2019). The astrocytes are important in the retention of the flavivirus in the CNS, in viral production and for spreading to neurons and other cells, and in the immunological response for microglial and activated T lymphocytes (Rock et al., 2004; Palus et al., 2014; Potokar et.al., 2019).

Microscopic lesions were consistently more severe in the midbrain, cerebellum, medulla oblongata and cervical spinal cord in the cluster B-severe lesions (severity grade III) (Salinas et al., 2017). However the quantification of the total area occupied by glial foci in the different regions of the brain revealed that medulla oblongata and all sections of the spinal cord were the most affected tissues. The IHC for microglia also statistically confirmed the lesion predominance on the right side of the medulla oblongata. That could be associated to the site (right) and route (subcutaneous) of SGEV challenge suggestive of a neurotropic route, as was hypothesized in the previous study by Salinas et al. (2017). In the former study histological lesions in thalamus and hippocampus were not detected, but were observed in the present study using Iba1-IHC. This technique is revealed to be a very useful tool for the detection of histopathological lesions otherwise difficult to detect using conventional hematoxylin and eosin staining.

**5. Conclusion**

The initial immune response to SGEV infection in goats would be established by the combined and balanced action of microglia and T lymphocytes suggesting and the B lymphocytes would likely increase throughout the infection time in response to the virus. The area occupied by glial foci revealed that medulla oblongata and spinal cord were the most affected tissues and that they should be the target tissues in sampling procedures.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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**Table 1. Immunohistochemical protocols used for cellular type characterization.**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Primary antibody** | **Company** | **Code/Clone** | **Specificity** | **Type of Antibody** | **Dilution** | **Epitope unmasking** |
| Iba11 | FLUJIFILM-Wako Chemicals Europe GmbH, Neuss, Germany | 019-19741 | Macrophages/microgial cells | Rabbit PAb3 | 1:1000 | Citrate pH 6.0 in microwave 20 min |
| CD3 | Novocastra, Leica Biosystem, Neucastle, United Kingdom. | NCL-L-CD3-565 | T lymphocytes | Mouse MAb4 | 1:500 | Citrate pH 6.0 in microwave 20 min |
| CD20 | ThermoFisher, Massachusetts, USA. | PA5-16701 | B lymphocytes | Rabbit PAb3 | 1:200 | Citrate pH 6.0 in steamer 20 min |
| GFAP2 | EncorBiotechnology, Gainesville, Florida, USA | MCA-5C10 | Astrocytes | Mouse MAb4 | 1:8000 | Citrate pH 6.0 in microwave 20 min |

1Iba1: ionized calcium binding adaptor molecule 1, 2GFAP: glial fibrillary acidic protein, 3PAb: polyclonal antibody; 4MAb: monoclonal antibody

**Table 2. Proportion (average of the percentages) of cell population types in goats experimentally infected with the Spanish goat encephalitis virus (SGEV).** Cellular phenotype (primary antibody) is shown according to the different levels of each of the main factors: cluster (A-moderate lesions corresponding to grades I and II and B-severe lesions corresponding to grade III) based on Salinas et.al. (2017), region of the nervous central system and type of lesion.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Variable** | **Level** | **Microglia (Iba1)** | | **T lymphocytes (CD3)** | | **B lymphocytes (CD20)** | | **Astrocytes (GFAP)** | |
|  |  | Mean | SE | Mean | SE | Mean | SE | Mean | SE |
| Cluster | A (moderate lesions) | 22.4 | 3.88 | 16.4 | 2.61 | 10.8 | 2.65 | 1.7 | 0.07 |
| B (severe lesions) | 53.1 | 3.47 | 26.5 | 2.33 | 22.8 | 2.37 | 1.8 | 0.07 |
| Region | Cortex | 31.2 | 7.33 | 15.1 | 4.92 | 17.8 | 5.00 | 1.6 | 0.14 |
| Thalamus, hypothalamus, corpus callosum | 53.1 | 7.33 | 32.6 | 4.92 | 21.3 | 5.00 | 2.2 | 0.14 |
| Hippocampus | 37.2 | 7.33 | 14.9 | 4.92 | 14.3 | 5.00 | 1.8 | 0.14 |
| Midbrain | 44.2 | 7.33 | 18.7 | 4.92 | 11.7 | 5.00 | 1.4 | 0.14 |
| Pons, cerebellum | 36.1 | 7.33 | 27.4 | 4.92 | 23.1 | 5.00 | 1.3 | 0.14 |
| Medulla oblongata | 49.6 | 7.33 | 30.1 | 4.92 | 19.1 | 5.00 | 1.8 | 0.14 |
| Cervical spinal cord | 29.8 | 7.33 | 22.9 | 4.92 | 16.2 | 5.00 | 1.8 | 0.14 |
| Lumbosacral spinal cord | 20.9 | 7.33 | 9.9 | 4.92 | 11.0 | 5.00 | 2.2 | 0.14 |
| Type of lesion | Glial foci | 38.4 | 3.68 | 24.9 | 2.46 | 14.3 | 2.50 | 1.8 | 6.97 |
| Perivascular Cuffing | 37.2 | 3.68 | 18.0 | 2.46 | 19.3 | 2.50 | 1.8 | 6.97 |

**Figure legends**

**Figure. 1. Representative immunohistochemical reactions for detection of microglia, T lymphocytes and B lymphocytes in thalamus (a, b and c) and medulla oblongata (d, e and f).** Avidin-biotin-peroxidase (ABC) complex method. Microglia (primary antibody Iba1): Numerous immunolabelled cells are noted both within a glial focus (a) and in the perivascular infiltrate of a vessel (d). Lymphocytes T (primary antibody CD3): Specific immunohistochemistry shows moderate numbers of T lymphocytes in the glial focus (b) and in the perivascular cuff (e). Lymphocytes B (primary antibody CD20): Few numbers of lymphocytes B are observed in the glial focus (c) and perivascular infiltrate (f).

**Figure. 2. Representative grades of reactive astrogliosis in cervical spinal cord (a, b and c) using the antibody against the glial fibrillary acid protein (GFAP) and the avidin-biotin-peroxidase (ABC) complex method.** (a) Grade 1: low level of astrogliosis is detectable. (b) Grade 2: mild to moderate reactive astrogliosis is present. (c) Grade 3: severe diffuse reactive astrogliosis is observed.

**Figure 3. Cluster analysis by the method of the centroid in the SAS CLUSTER procedure.** Two clusters are clearly defined based on the previous study Salinas et al. (2017). These clusters were considered as overall microscopic lesions of three levels; grade I and II, goats 78, 79, 80 and 82 into cluster A (moderate lesions) and grade III, goats 76, 77, 81, 85 and 86 into cluster (B-severe lesions).

**Figure. 4. Scanned image of the entire cross-section of the medulla oblongata.** Detection of microglia (antibody Iba1) using the avidin-biotin-peroxidase (ABC) complex method. Note the presence of more glial foci on the right side of the tissue. Inset: detail of the affected parenchyma.