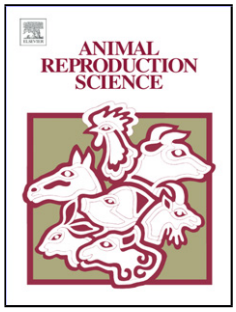
Expression of adenosine 5'-monophosphate-Activated protein kinase (AMPK) in ovine testis (Ovis Aries): In vivo regulation by nutritional state

N. Taibi, Joëlle Dupont, Z. Bouguermouh, Pascal Froment, Christelle Ramé, A. Anane, Z. Amirat, F. Khammar



Title: Expression of adenosine 5′-monophosphate—Activated protein kinase (AMPK) in ovine testis (O*vis Aries*): *In vivo* regulation by nutritional state

Authors: N. Taibi, J. Dupont, Z. Bouguermouh, P. Froment, C. Ramé, A. Anane, Z. Amirat, F. Khammar

PII: S0378-4320(16)30332-3
DOI: http://dx.doi.org/doi:10.1016/j.anireprosci.2017.01.003
Reference: ANIREP 5525

To appear in: *Animal Reproduction Science*

Received date: 11-8-2016
Revised date: 4-1-2017
Accepted date: 7-1-2017

Please cite this article as: Taibi, N., Dupont, J., Bouguermouh, Z., Froment, P., Ramé, C., Anane, A., Amirat, Z., Khammar, F., Expression of adenosine 5′-monophosphate—Activated protein kinase (AMPK) in ovine testis (Ovis Aries): In vivo regulation by nutritional state.Animal Reproduction Science http://dx.doi.org/10.1016/j.anireprosci.2017.01.003

Expression of Adenosine 5'-Monophosphate –Activated Protein Kinase (AMPK) in ovine testis (O*vis Aries*): *in vivo* regulation by nutritional state

N. Taibi [a,b*], J. Dupont [c], Z. Bouguermouh [d], P. Froment [c], C. Ramé [c], A. Anane[a], Z. Amirat [a], F. Khammar [a]

Corresponding author:

Phone: + 213 561 282 486/ E-mail address*: nadbio71.doc@gmail.com

E-mail address: Jdupont@tours.inra.fr

E-mail address: Zahir.bouguermouh@nutristar.fr

E-mail address: Pascal.froment@tours.inra.fr

E-mail address: Christelle.rame@tours.inra.fr

E-mail address: anane_assia@yahoo.fr

E-mail address: amiratzaina@yahoo.fr

E-mail address: faridakhammar@gmail.com

a University Science Technology Houari Boumediene (USTHB), FSB, Laboratoire de Recherche sur les Zones Arides, (LRZA), BP 44 Alger-Gare, 16000, Algérie.

b Centre de Recherche Scientifique et Technique en Analyses Physico-chimiques (C.R.A.P.C), BP 384, zone industrielle de Bou-Ismail, RP 42004 w. Tipaza Algérie

c Unité de Physiologie de la Reproduction et des Comportements, Institut National de la Recherche Agronomique, 37380 Nouzilly, France.

d Nutristar International Group CCPA

HIGHLIGHTS

- p-AMPK was identified in Leydig cells even in the presence of high ATP levels,
- Nutritional phases have induced an isoforms-dependent AMPK with an increase in α2, ß1ß2 and γ1,
- Restriction diet had no effect on p-AMPK expression in liver,
- High level of lipoproteins according to restriction diet probably induce IL-1 that act as transcriptional repressors of the StAR protein,
- Highlighting two signaling pathways p38 and p44 / 42 MAPK.
- AMPK expression is tissue-dependent

Abstract

In the present study, we identified AMPK and investigated its potential role in steroidogenesis *in vivo* in the ovine testis in response to variation in nutritional status (fed control vs. restricted). We performed immunoblotting to show that both active and non-active forms of AMPK exist in ovine testis and liver. In testis, we confirmed these results by immunohistochemistry. We found a correlation between ATP (Adenosine-Triphosphate) levels and the expression of AMPK in liver. Also, low and high caloric diets induce isoform-dependent AMPK expression, with an increase in α2, ß1ß2 and γ1 activity levels. Although the restricted group exhibited an increase in lipid balance, only the triglyceride and HC-VLDL (Cholesterol-Very low density lipoprotein) fractions showed significant differences between groups, suggesting an adaptive mechanism. Moreover, the relatively low rate of non-esterified fatty acid released into the circulation implies re-esterification to compensate for the

physiological need. In the fed control group, AMPK activates the production of testosterone in Leydig cells; this is, in turn, associated with an increase in the expression of 3ß-HSD (3 beta hydroxy steroid deshydrogenase), p450scc (Cholesterol side-chain cleavage enzyme) and StAR (Steroidogenic acute regulatory protein) proteins induced by decreased MAPK ERK½ (Extracellular signal-regulated *kinase* -Mitogen-activated protein kinase) phosphorylation. In contrast, in the restricted group, testosterone secretion was reduced but intracellular cholesterol concentration was not. Furthermore, the combination of high levels of lipoproteins and emergence of the p38 MAP kinase pathway suggest the involvement of pro-inflammatory cytokines, as confirmed by transcriptional repression of the StAR protein. Taken together, these results suggest that AMPK expression is tissue dependent.

Keywords: domestic ruminant; diet; steroidogenesis; AMPK; signal transduction

1. Introduction

Steroidogenesis is an important process in spermatogenesis and consequently in male fertility. It is dependent upon the Leydig cells that produce androgens including testosterone that maintain male reproductive function. Acute and chronic regulations of steroidogenesis are controlled by hormones subjected to changes in cellular energy and cholesterol homeostasis. Whereas, chronic responses involve the increased transcription of the genes encoding the steroidogenic enzymes (Simpson and Waterman, 1988), acute responses to hormonal stimulation require *de novo* protein synthesis (Clark et al., 1994). Early studies conducted by (Anderson and Mendelson, 1985) also demonstrate that treatment of immature porcine or rat testicular cells in culture by hCG and/or cAMP increases *de novo* synthesis of p450scc and associated proteins. Steroid hormones regulate essential physiological processes, and inadequate levels are associated with various pathological conditions (Abdou et al.,

2014). In fact, it has become clear that the regulation of the steroidogenic enzymes is a complex process and is closely dependent of diet. For examples, it was reported that the testicular 3ßHSD protein expression could be influenced by dietary lipid composition (Rasmussen et al., 2013).LH (Luteinizing Hormone) secreted from cells in the anterior pituitary stimulates secretion of sex steroids from the gonads. In the testes, LH binds to receptors on Leydig cells, stimulating synthesis and secretion of testosterone. At the molecular level, LH triggers a series of intracellular changes mediated mainly by the activation of cAMP-dependent protein kinase A. This latter process can be reduced at any time by nutritional conditions. Food restriction can lead to a depletion of ATP in the cell and mitochondrial fatty acid oxidation. One of the central regulators of cellular metabolism in eukaryotes is the AMP-activated protein kinase (AMPK), which is activated when intracellular ATP levels lower. AMPK is also considered as a molecular rheostat that actively represses steroid hormone biosynthesis to preserve cellular energy homeostasis and prevent excess steroid production (Abdou et al., 2014).

In most species, AMPK exists as a heterotrimer, containing a catalytic subunit (α) and two regulatory subunits (β and γ). The activation of AMPK occurs by allosteric and covalent modifications of the enzyme in response to an energetic deficit (Kahn et al., 2005). In mice, α1AMPK deficiency in male affects androgen production and quality of spermatozoa, leading to a decrease in fertility (Tartarin et al., 2012). Moreover, disruption of α1AMPK in mice specifically in Sertoli cells translates to a reduction in the quality of germ cells and fertility. In testis, Sertoli cells are absolutely necessary in order to provide an adequate and protected environment within the seminiferous tubules. *In vitro* Galardo et al. 2007 showed that AMPK activation increases lactate production suggesting that an important role of AMPK in modulating the nutritional function of Sertoli cells (Galardo et al., 2007). AMPK is also expressed in germ cells and it controls the quality of sperm by its involvement in the

regulation of motility and acrosome reaction in different species including chicken and pig (Nguyen et al., 2014), (Hurtado de Llera et al., 2016), (Hurtado de Llera et al., 2015). In human, AMPK is present in the seminiferous epithelium and interstitium of testis and is expressed in a differential and developmental manner in particular cell types (Ham et al., 2016). However, AMPK has never been characterized in vivo in ovine testis.

The aims of our study were to demonstrate the presence of AMPK in ovine testis and to investigate its expression *in vivo* in response to dietary restriction. We also investigated the role of AMPK in testosterone production induced by diet composition.

2. Materials and methods

2.1. Hormones and reagents

[1, 2, 6, 7-^{3}H] testosterone (sp. act. 75 Ci/ mM) was purchased from Radiochemical Centre (Amersham, Great Britain). Non-labelled testosterone was obtained from Sterloids (Wilton, NH, USA). Testosterone anti-serum was a gift of G. Picaper (CHR, Orléans, France). It was raised against testosterone 3-O-carboxymethyloxime-BSA and its affinity constant was 2.5 x 10^{8} M^{-1}. The anti-rabbit γ-globulins sheep serum was prepared by Dr. M. Blanc (INRA, Nouzilly, France). Polyethylene Glycol was purchased from (Sigma, Aldrich). The cAMP-Glo™ Kit Assay was purchased from (Promega, Madison, WI, USA).

2.2.Antibodies

2.2.1. Primary antibodies

Rabbit polyclonal antibodies to phospho-AMPK α (Thr 172)40H9 Rabbit mAb (#2535), AMPK ß½ (57C12) Rabbit mAb (#4150), AMPK γ3 (#2550), AMPK γ1 (#4187), phospho-p38 kinase (Thr180/Tyr182)/ (#9211), phospho-p44/42 MAPK (Erk½) (Thr202/Tyr204)/(#9101), and p44/42 MAPK (Erk ½)/ (#9102) were purchased from Cell Signaling Technology®. Rabbit polyclonal IgG to Anti-AMP-Activated Protein Kinase

(AMPK) α1 (# 2749) was purchased from Upstate, EUROMEDEX). Rabbit polyclonal antibodies to AMPK α1 (C-20): sc-19128, p38α (C-20): sc-535 were purchased from SANTA CRUZ BIOTECHNOLOGY, INC. Monoclonal Anti-Vinculin antibody (produced in mouse) was purchased from SIGMA-ALDRICH, St. Louis MO 63103 USA.

2.2.2. Secondary antibodies

HRP goat anti-rabbit IgG and HRP goat anti-mouse IgG secondary antibodies were used to detect the primary antibodies listed above. All antibodies were used at 1/1000 dilution in western blotting and 1/100 dilution for immunohistochemistry. All these antibodies have been previously validated in sheep species (Scaramuzzi et al., 2015) ; (Gallet et al., 2011)

2.3 Animals

Adult male Ouled–Djellal sheep (OD) (6 months old) were purchased from a governmental farm. All procedures were approved by the Agricultural and the Scientific Research Agency, and conducted with the Ministry of Agriculture and Rural Development in accordance with the Directive of the 2010/63/EU animal experiments; (http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm). Animals were kept in a temperature-controlled room under natural photoperiod with water available *ad libitum* and food in accordance with experimental protocol. Ouled–Djellal sheep were randomly assigned to tow groups (control vs. restricted). The control (n=3) and the restricted (n=4) groups received 1000 vs. 400 grams/day/animal, respectively a mixture formulated by experts in animal nutrition. The main experience was conducted during the breeding season.

2.4 Experimental ration

The diet for growing lambs was adjusted to 1 kg / day / animal. This formulation was a gift from Dr. Z. Bouguermouh, Nutristar International-Algeria. The feed consisted of 65% maize, 10% wheat bran, 22% of soya, 2% of carbonate calcium, 1% salt and 1% CMV (NUTRISTAR sheep). The assessment of the nutritional component of the ration showed a

rate of 3.13% fat, 47% starch, 3.5% cellulose, 17% protein, 0.23% methionine, 0.81% lysine, 1.20% calcium and 0.41% phosphorus.

2.5 Animal Treatment

At the time of purchase, all male Ouled–Djellal sheep weighed (13.00 ± 0.18 kg). For the purposes of the experiment, the sheep were housed in individual boxes in minimizing stress environment. All animals were subjected to an adaptive intake (20 % of total ration i.e. 1000 vs. 400 grams/day/animal) during 3 weeks. This rate was calculated to maintain satisfactory body condition and avoiding malnutrition. By the fourth week, the experimental protocol was initiated, sheep therefore weighted 23.61 ± 0.57 vs. 20.54 ± 0.59 kg for control and restricted groups respectively ($P < 0.0001$). Lambs were weighed and measured (height at withers, thoracic girth and scrotal circumference) once a week before the meal 09:00 a.m. during 15 weeks. At the end of experiment, they were sacrificed; blood was collected to measure biochemical parameters (glucose, total protein, triglycerides, total cholesterol, HC-HDL, HC-LDL, HC-VLDL, NEFA and total lipids). Liver and testis samples were surgically removed, defatted, released of their adhesions and rapidly immersed in liquid nitrogen and stored at - 80 ° C before RNA (Ribonucleic acid) and protein isolations. A part of testis was fixed in neutral buffered formalin 10% for immunohistochemistry.

2.6 Biochemical parameters (Spectrophotometry)

2.6.1 Glucose [Code No: BSIS 46-I, Method: GOD-POD. Liquid, Packaging Ref: 41013, Storage: 2-8°C], total proteins [Code No: BSIS 30-1, Method : Biuret Colorimetric, Packaging Ref : 1001292, Storage : 2-8°C], triglycerides [Code No: BSIS 49-1, Method: GPO-POD. Enzymatic Colorimetric, Packaging Ref: 1001313, Storage: 2-8°C], total cholesterol and HC-HDL [Code No : BSIS 11-1, Method : CHOD-PO. Enzymatic Colorimetric, Packaging Ref: 1001093, Storage: 2-8°C]. Biochemical parameters levels are estimated by a general protocol for colorimetric assay (SPINREACT Kits, S.A/S.A.U Ctra. Santa Coloma, 7 E-17176 SANT ESTEVE DE BAS (GI) SPAIN.). Biochemical parameters levels are estimated by a general

protocol for colorimetric assay (SPINREACT Kits, S.A/S.A.U Ctra. Santa Coloma, 7 E-17176 SANT ESTEVE DE BAS (GI) SPAIN.).

2.6.2 Wako NEFA-HR Kit (2) [Code No: 436-91995, Method: ACS-ACOD-MEHA, Packing Size: R2: 4 x 25 ml, Storage: 2-10 ° C] uses an enzymatic method for the Quantitative determination of non-esterified fatty acids (NEFA-HR (2)) in the serum sold by Wako Chemicals GmbH-Laborchemikalien - Wako Gruppe - AGB.

The quantitative determination of the parameters listed below was evaluated using colorimetric assay. The color intensity is proportional to the concentration of analytes assayed. The intra- and the inter assay coefficient variation were 10.07 and 13.57 % respectively.

HC-LDL, HC-VLDL and total lipids were calculated according to the formulas cited below:

LDL-Cholesterol (g / L) = (Total Chol) - (HDL-Chol) - (Triglycerides) / 5 (Friedewald et al., 1972).

VLDL-Cholesterol (g / L) = (Total Chol.) - HDL-LDL

Total Lipids (g / L) = (Total Cholesterol) x 2.56 + (Triglycerides) x 0.87

2.7. Steroid production

The concentration of testosterone was measured directly without extraction over the range of 0.03 to 16 ng/ml in fresh testis frozen at -80°C using a radioimmunoassay protocol as described previously (Hochereau-De Reviers et al., 1990). The assay sensitivity was 15 pg/ml, the limit of detection of testosterone was 3.1 pg/tube, and the intra- and the inter assay coefficient variation were 4.1 and 13% respectively.

2.8. AMPc production

Cyclic AMP was measured by luminescence using reagents prepared in our laboratory according to the manufacturer's instructions (Promega, Madison, WI, USA). For these assays, samples were prepared from frozen fresh testicular tissue by homogenization in 20 µl of lysis

buffer, 40 µl of detection solution and 80 µl of Kinase-Glo® reagent. All of these reaction steps require incubation times between 10 and 20 min at room temperature. The generating cAMP Standard Curve was performed prepared from a concentration of 4.0 µM for a range set of 0.0078 to 0.0156 µM.

2.9 Cholesterol determination

The quantitative determination of total Cholesterol in sheep testis uses a colorimetric assay kit (Enzymatic CHOD-PAP method) purchased from Biolabo–SA, Maizy, France under reference Cat N0:80106.

2.10. Immunohistochemistry identification of AMPKα, AMPK α1, AMPKß1ß2, p-AMPK α and anti-AMPKα1

Testes embedded in paraffin were serially sectioned at a thickness of 7 µm. Sections were deparaffinized, hydrated in baths as increasing alcohol and microwaved at boiling for 2 min in antigen unmasking solution diluted at 1:100 (sodium citrate solution 10 mM, pH 6.0) (Vector Laboratory, Inc., AbCys, Paris, France). After cooling, slices were washed in Phosphate Buffered Saline (PBS) bath for 5 min and then exposed to a 0.3 % hydrogen peroxide solution for 20 min at room temperature to block the activity of endogenous peroxidase (Kit Dako). The immunodetection step required the saturation of nonspecific background by blocking them with 5% of horse serum in PBS for 30 min following by incubation overnight at 4°C with rabbit polyclonal primary antibodies (1:100; dilution of phosphor-APMK α (Thr172), (Cell Signaling Technology®, # 2535) and AMPK α1 (C-20):sc-19128 Santa Cruz Biotechnology, INC). After they were washed twice in PBS for 5 min each time, they were incubated for 30 min at room temperature with labelled polymer-HRP-anti-rabbit (Dako, Envision plus System-HRP (DAB)). The visualization of the staining was accomplished by using tetrachlorure 3, 3'-diaminobenzidine (DAB) (Vector Laboratories, Vectastain kit). The slides were then counterstained with Meyer's hematoxylin (Sigma)

before mounting. IHC controls included omission of the primary antibody from immunostaining protocol was voluntarily replaced by Rabbit polyclonal antibody IgG.

2.11 Protein extraction and western blotting

Testes tissues were homogenized in 3 ml of lysis buffer [(10 mM Tris Base (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA and 0.5% Igepal)] containing a protease inhibitor cocktail (2 mM phenylmethylsulfonyl fluoride, 10mg/ml leupeptin, and 10mg/ml aprotinin) and phosphatase inhibitors [100 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate (Sigma, l'Isle d'Abeau Chesnes, France)]. Lysates were then centrifuged at 16 000 g x 30 min at 4°C. The supernatant was collected and protein concentration was determined by BCA assay (Kit; Uptima Interchim, Montluçon, France). Samples proteins were separated by SDS-PAGE gel under reducing conditions and then electrotransferred onto nitrocellulose membrane for 2 h at 80-90 Volts. The membranes were blocked in Tris-buffered saline (TBS) (2 mM Tris-HCl, pH8.0; 15 mM NaCl, pH7.6) with 5% milk powder and 0.1% Tween 20 and incubated overnight at 4°C in primary antibody (diluted 1:1000 for p-AMPK, AMPK α, AMPK γ1, AMPK ß½, p38α, phospho-p38 Kinase, p44/42 MAPK (Erk½), Phospho-p44/42 MAPK (Erk½)). After washing in TBS, membranes were incubated at room temperature for 1h30 min in HRP-conjugated secondary antibody (1:5000). Proteins were revealed by enhanced chemiluminescence (Western Lightning Plus-ECL, Perkin Elmer) using a G:Box SynGene (Ozyme) with GenSnap software (release 7.09.17). Quantification was performed with GeneTools software (release 4.01.02). Protein relative abundance was quantified and normalized against Vinculin or ß-actin as reference proteins.

2.12 RNA isolation and Quantitative RT-PCR

Total RNA was isolated from tissues using TRizol reagent (Invitrogen^{TM} Life technologies, Carlsbad, CA) according to the manufacturer's protocol. RNA purification was

achieved by treating samples with DNAse followed by the use of RNeasy Mini Kit (Qiagen, Hilden Germany) and RNA concentration was quantified by measuring absorbance at 260nm. To generate cDNA, 2µg of total RNA were denatured and reverse-transcribed for 1 hour at 37 °C in 20 µl of reaction mixture containing 50 mM Tris-HCL (pH 8.3), 75 mM KCL, 3 mM $MgCL_2$, 200 µM of each deoxynucleotide triphosphate (Amersham), 50 pmol of Oligo (dT), 15.5 IU of ribonuclease inhibitor and 15 IU of Moloney murine leukaemia virus reverse transcriptase. *After reverse transcription, the cDNAs of ovine tissues were diluted 1:5. Real-time PCR was performed in a 20 µl final volume containing* 10 µl iQ SYBR Green supermix (Bio-Rad), 0.25 µl of each primer (10 µM), 4.5 µl of water and 5 µl of template. The cDNA templates were amplified and detected with the MYIQ Cycler real time PCR system (Bio-Rad) using the following protocol: 1 cycle for 5 min at 95°C to denature the sample and then 40 cycles, 1 min at 95°C for denaturation, 1 min at 60°C for hybridization, 1 min at 72°C for stretching and finally 1 cycle for 5 min at 72°C for final elongation. Gene expression was normalized using the geometric mean of three housekeeping genes (UXT, SDAH and CYCLOA) with stable expression under our conditions (data not shown).The description of the different primers (housekeeping genes and target genes (3betaHSD, StAR and P450scc) is indicated in Table 1. The normalized values of relative expression (R) were calculated according to the following equation: $R= : (E_{gene}\text{-}^{CTgene})/(\text{geometric mean } (E_{UXT}\text{-}^{CTUXT} ; E_{SDAH}\text{-}^{CTSDAH} ; E_{CYCLOA}\text{-}^{CTCYCLOA)})$, where CT is the cycle threshold and E is the PCR efficiency for each primer pair. The specificity of the amplified fragment sequence was assessed by Beckman Coulter Genomics (Essex, United Kingdom). The efficiency was between 1.7 and 2.

2.13. Statistical analyses

Statistical analysis of the biological data was performed using one-way ANOVA (Tukey's multiple comparison tests) on XLSTAT 2014 software to compare states of feeding

and the expression of total and phosphorylated AMPK. All results were considered significant at $P \leq 0.05$.

3. Results

3.1. Combined effects of control and restricted diets on changes in anthropometric and metabolic parameters.

The changes in feed intake during this period in OD sheep induced a notable difference in the expression of anthropometric and biochemical indicators, as summarized in Table 2. Body weight (BW), height at the withers (HW), thoracic perimeter (TP) and scrotal circumference (SC) were higher in control group as compared to restricted group. However, no significant difference was observed between the two experimental groups for the nutritional variant as was the case with age ($P < 0.0001$). Concerning biochemical parameters, plasma glucose was lower whereas total protein, triglyceride, cholesterol, HC-VLDL, HC-HDL, HC-LDL and total fat were higher in restricted than control animals. Only triglycerides ($P < 0.01$) and the HC-VLDL lipoprotein fraction ($P < 0.01$) showed a statistically difference between the two groups for nutritional phases proposed.

3.2. *In vivo* immunoblotting identification of subunit structure of AMPK in both testis and liver tissues.

We performed immunoblotting to identify the three subunits of AMPK: α, ß and γ. in both testis (Fig. 1A, 1B, 1C) and liver (Fig. 1A', 1B', 1C') Analysis of electrophoretic transfer showed one band corresponding to the AMPKα (63kDa) and AMPKγ1 (37kDa) and two bands corresponding to AMPKß1/ß2 (38 and 30kDa), respectively. Vinculin and ß-actin were used as a loading control.

3.3. Effect of nutritional state on the expression of both AMPK and Thr 172 p-AMPK in the testis revealed by immunohistochemistry

As shown in Fig. 2, microscopic analysis of testicular sections revealed lower expression of AMPK phosphorylation levels in the control group as compared to the restricted group. Similar data were obtained with the total AMPK protein antibodies (Fig. 3). Although the concept of immunohistochemical labelling allowed demonstrating a co-localization of both AMPK and p-AMPK proteins in sheep testis, it unfortunately failed to accurately assess the importance of immunostaining during the nutrition conditions proposed, hence the interest to confirm this step by western blot.

3.4. *In vivo* quantification of AMPK and Thr 172 AMPKα-phosphorylation expression by immunoblotting

First, we investigated the impact of control and restricted diets on the expression of both phosphorylated and total AMPK α in hepatic and testicular tissues. As summarized in Fig.4, there is an inequality in the expression of AMPK levels between the two nutritional states in both liver and testis. Although no significant difference (P> 0.05) was observed for the AMPK between the two groups, there is nevertheless a clear increase of its testicular level (39%) (Fig. 4A) compared with liver (4%) (Fig. 4A'). Then, we measured possible changes in the expression of p-AMPK α (Th-172). As depicted in Fig. 4B, testicular p-AMPK is widely expressed in the restricted group, an increase of more than two-fold is observed as compared to control group. Unlike the testis, the liver shows a decrease of p-AMPK rate for restricted group Fig. 4B'. Our results show that phosphorylation of AMPK is differently regulated in liver and testis in response to a restriction.

In our animal model, we have established that there was an effect of food intake on the abundance of both total AMPK and p-AMPK α in liver and testis, but these trends were not significant. However, there was a statistically significant effect of food intake on the ratio p-AMPK/AMPK α which was lower in testis (p=0.091); Fig.4C and higher in liver (p=0.05); Fig. 4C' in the restricted compared with the control group.

3.5. Evaluation of testicular production: testosterone levels and generation of cAMP

As shown in Fig. 5A, a stimulatory effect of food on testosterone production was observed in the control as compared to the restricted group (6.39 ± 3.22 vs. 0.79 ± 0.27 ng/ml). Additionally, we find that the Control group appears more efficient since an increase by 88% is noted ($P < 0.05$). Concerning cAMP the results suggest a relatively small increase (5,97E+05± 2,34E+05 vs 5,80E+05 ± 1,15E+05 µM) between the two groups Fig. 5B . We find that the profiles of testosterone and cAMP evolve synchronously in favour of the Control group. In addition, assays performed on testicular extracts showed that cholesterol levels were slightly higher in the restricted as compared to the Control group (0,79 ± 0.02 vs. 0,77 ± 0.01 mM), a noticeable increase is observed between the two groups Fig. 5C. Next, we investigated the possible existing correlations between the source of cholesterol and the final product "testosterone". Unfortunately, no correlation was observed excepted for the couple (steroidogenic acute regulatory protein; StAR) StAR/HC-LDL ($r=0.78$; $P=0.037$). Fig. 5D.

3.6. Effect of the nutritional state on the expression of StAR, P450scc and 3ßHSD proteins in Control and Restricted-Fed Phases in sheep testis

We next examined the impact of nutritional states on the mRNA expression of key proteins required for testosterone production (two key enzymes of steroidogenesis (3β-HSD and P450scc) and the important cholesterol carrier (StAR) in testis. As shown in Fig. 6, there was a tendency that the 3ßHSD messenger was highly expressed in restricted as compared to control group ($p=0.07$). At the opposite, there was no statistically significant difference in the amount of StAR and P450scc ($P>0.05$) between the two groups. Based on the ratio of housekeeping gene, Cyclo A, SDHA and UXT, the StAR protein expressions was decreased by 61 % in restricted as compared to control group (155 %) while a significant raised was observed for both p450scc (+11%) and 3ßHSD (+51%).

3.7. Effect of the nutritional states on the regulation of p44/p42 (EKR1/2) and p38 MAP-Kinase signalling pathway.

In order to follow the chronology of events in the testes, we focused on the activation of MAPK signalling pathways (p44/p42 and p38) involved in the steroidogenic process (Tosca et al., 2005). First, the immunoblotting results showed an overexpression of p44/p42 MAPK in the control group for both total and active form. Arbitrary quantification of p44/p42 MAPK confirmed an increase by 97% of the total p44/p42 MAPK in Control against a decrease by 60% of the active form in the restricted group. In testis, total p44/p42 was increased in control group as compared to restricted group (Fig. 7A, 7B: P=0.05). At the opposite no significant effect was observed between the two group concerning p44/p42 MAPK phosphorylation (Fig. 7C, 7D: P=0.09). Proportion of phosphorylation to total p44/p42 MAPK (ratio) was also evaluated and found no significant difference between the two groups (Fig. 7E). Next, we examined the expression of p38 MAPK protein level in the proposed experimental conditions. Active (Fig. 7A') and phosphorylated (Fig.7B') forms of p38 MAPK were higher in control and restricted group respectively. We also showed that the ratio of pp38:p38 MAPK in Restricted group was higher (p=0.06) than in Control group (Fig. 7E').

4. Discussion

The purpose of the current study was to investigate the effect of nutritional status on steroidogenesis and the expression of AMPK in ovine testis. In both experimental diets (control and restricted) we demonstrated by immunohistochemistry the presence of AMPK and its phosphorylated form in the testes specifically in Leydig cells. Once activated in response to both nutrient deprivation (Salt et al., 1998) and environmental stress (Choi et al., 2001), it senses a priori changes in the ratio of AMP: ATP by binding both species in a competitive manner (Viollet et al., 2003a). It is admitted that AMP-activated protein kinase is

recognizing such as an ultra-sensitive system for monitoring cellular energy charge (Hardie et al., 1999). Given the importance of AMPK in regulating glucose metabolism, the identification of the three subunits in the holoenzyme complex (α_2, $ß_1ß_2$ and γ_1) by immunoblotting was seemed necessary. In Control group, AMPK is largely inactive, present in the dephosphorylated form and following glucose deprivation initiated in the restricted group, we suppose generating an AMP sensitive complex. Hardie and Ashford provided evidence that low concentration of AMP caused a great allosteric activation in the cell free assay, even in the presence of ATP much higher (Hardie and Ashford, 2014). This evoked the possibility that AMPK might be sensitive to submicromolar concentration of AMP (Suter et al., 2006).

In the present study, we showed that the level of AMPK phosphorylation is higher in testes than in liver. This disparity suggests that activation of AMPK is reduced in tissue containing high levels of glycogen (Wojtaszewski et al., 2002) and propose that caloric restriction decreases AMPK activity in the liver over the long term as described by (To et al., 2007). Low and high caloric diet have been shown to induce an isoforms-dependent AMPK, with an increase in α2 activity confirmed by liver and testes immunoblotting. To check the direct involvement of AMPK α2 in the regulation of glucose homeostasis, (Viollet et al., 2003b) demonstrate that a transgenic mice would exhibit both glucose intolerance and reduce insulin sensitivity in peripheral tissue confirming that AMPK α2 hepatic isoform is essential to suppress hepatic glucose production and maintain fasting blood glucose levels in the physiological range (Kanoh et al., 2001). In the present study, reduction in plasma glucose observed in the restricted group in response to energy deficit below the physiological range is strongly associated with a decrease in endogenous hepatic gluconeogenesis supported by energy sensing systems including AMPK, SIRT1, LKB1, ACC and TORC2 (Shoba et al.,

2009). For instance, an increase in hepatic gluconeogenesis in the Control group that is nothing unusual in ruminants, with slightly elevated blood glucose would probably depend on the duration of the overnutrition (Yoneda et al., 2010).

We showed expression of different forms of AMPK (α2, ß1ß2, γ1) in both testis and liver in ovine species. Notably, these isoforms monitor energy availability by sensing ATP and also by sensing the status of cellular energy stores in the form of glycogen. In fact, the sheep liver glycogen structure may be differing in their ability to inhibit AMPK because of both length of the carbohydrate chain and also degree of branching referring to nutritional states. It was reported that the glycogen molecule has theoretical maximum of 12 tiers of branching (Melendez-Hevia et al., 1993) and between 12 and 18 glucose units (Illingworth et al., 1952). As observed with a synthetic branched oligosaccharides (McBride et al., 2009) the presence of a free reducing end abolishes the inhibition of AMPK whereas the trapping of the non-reducing end of the chain containing a single α1-6 branch enhances the inhibitory power on AMPK. The architectural conformation such presented above with its different ligands allow the reverse lock of the glycogen molecule and the recruitment of many proteins (glycogen phosphorylase, glycogen synthase, phosphatase, and now AMPK) (Shearer and Graham, 2002) may be driven by the outer chain involved in either glycogenolysis or gluconeogenesis pathway. Our results reinforced the hypothesis that glycogen could inhibit AMPK and that ß-subunit is an allosteric activator of AMPK and explain the disparity observed between the metabolic and reproductive tissue.

AMPK is also known to stimulate fatty acid oxidation in liver cells (Kahn et al., 2005) in response to energy depletion. In Ouled–Djellal, we observed a negative correlation between the availability of circulating glucose and plasma HC-VLDL (Table 2) that is consistent with the work of (Zenimaru et al., 2008) suggesting that the VLDL receptor pathway might be one of the mechanism by which the cells obtain fatty's acids TG-rich lipoproteins. This adaptation

is necessary to restore glucose deprivation. In fact, during food restriction, the glucose deprivation (Table 2) induces a change in the main energy substrate from glucose to fatty acids which explains the relatively high plasma NEFA (*257.40 ± 22.45* vs. 307.71 ± 97.45 mM/l) in the restricted-feed Group. In made during fasting, 30-40% of NEFAs released from TG stores are reesterified (Reshef et al., 2003), providing a mechanism to limit NEFA release into the circulation. Together, these results suggest that PKA-mediated phosphorylation of AMPKα1 represents a critical and relevant mechanism contributing to the regulation of lipolysis *in vivo* (Djouder et al., 2010).

Our study also focuses on existing lipoprotein variants. We note that the lipid profile (HDL-HC, HC-LDL, total cholesterol and triglycerides) was abnormally high in the restricted-feed (Table 2). One hypothesis is that the increase in plasma lipoproteins observed in restricted group is necessarily due to unbalance in diet composition. In fact, recent study conducted on Human volunteers showed that energy-restricted high-carbohydrate diet resulted in a decrease in HC-HDL (Turley et al., 1998). Furthermore, it is obvious that increasing the feed of the lipid content changes the composition of plasma lipoproteins, including increasing the amount of cholesterol esters in the VLDL, LDL and HDL confirmed by (Fernandez and West, 2005). These changes in the composition of lipoproteins are related to increased activity of the hepatic 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMG-CoA), hepatic ACAT (Acyl-CoA Cholesterol Acyl Transferase) and plasma LCAT (Lecithin Cholesterol Acyl Transferase). In addition, several studies conducted *in vivo* have demonstrated that the combination of high fat and simple carbohydrate resulted in elevated plasma LDL, cholesterol concentrations and high level of plasma triglycerides (McNamara, 1992). According to (Fernandez and West, 2005), different types of fatty acids can also modulate plasma cholesterol concentrations. In fact, saturated fatty acid rich-diet rather than PUFA (Poly-unsaturated Fatty Acids) stimulates the transcription of lipogenic genes **[**Glycerol-3-phosphate acyltransferase **(**GPAT**)**, Fatty acid synthase **(**FAS**)**, Acetyl-coenzyme A carboxylase **(**ACC**)]** by either stimulating SREBP-1 (Sterol regulatory element-binding protein) gene transcription or by enhancing the maturation of SREBP-1 protein. In ruminants, these lipogenic enzymes can be downregulated in response to restricted dietary intake (Bauman et al., 2006) by competing directly between glycerol-3-phosphate acyltransferase (GPAT) and carnitine palmitoyl transferase-I (CPT1) activity for triglycerides and fatty acids synthesis.

Having highlighted the different subunits of AMPK in ovine testis, we investigated the potential role of this kinase on testicular steroid production and the signalling pathways

involved. The reproductive aspect is interesting because it implicitly allowed emphasizing that the deprivation of glucose affected the pulsatility of LH and thus the levels of testosterone. The deleterious effects of caloric restriction could have consequences on the reproductive function and more precisely on the expression of molecules involved in the steroidogenesis such as StAR, p450scc and 3ßHSD. Indeed, exposure to chronic LDL levels observed in restricted group (Table 2) caused by unbalanced energy intake could be sufficient to trigger a cellular response involving interleukin-1α (Zhu et al., 1999). The latter could in turn modulate the Leydig cell membrane functions by decreasing the LH / HCG receptors (Mauduit et al., 1992) and activating the transcriptional repressor of steroidogenic enzyme gene expression of StAR (Hales, 2002).

Given a relatively high presence of intra-testicular cholesterol, we wanted to understand its origin. The 25-hydroxycholesterol produced by testicular macrophages after enzymatic conversion of cholesterol (Lukyanenko et al., 2001) reinforces the idea of a pool of naturally occurring sterols (de novo cholesterol synthesis from acetate). In our hypothetical approach, we have targeted a probable failure of the mitochondrial enzymatic system responsible for the transfer of cholesterol. (Abdou et al., 2014) clearly demonstrate this by the passive translocation of OH-cholesterol into the mitochondrial internal membrane, which places the problem at any other level. Treatment of MA-10 and MLTC-1 cells by Forskolin, an Adenylate cyclase activator, resulted in increased protein levels of StAR, Nr4a1 (transcriptional activator), Scarb1 (Scavenger Receptor 1) mRNAs, All are abolished by the activation of AMPK (Tosca et al., 2005). These data indicate that AMPK targets the expression of the StAR gene and responds to the collapsed level of StAR in restricted sheep.

Presumably, the main way of regulation of steroidogenesis is through the stem Hypothalamo-pituitary via LH and cytokines. The possibility that the expression of Nur77 (Nr4a1) is regulated by IL-1α has been demonstrated by El-Asmar et al, 2008. Indeed, IL-1α is known to activate at low-dose steroidogenesis (Renlund et al., 2006) and to inhibit it at high

dose levels in Leydig cells. Confirmed results on primary cultures of immature rat Leydig cells treated with 20 µg/ml of IL-1α for four hours. A 6 to 7-fold increase in the level of the Nur77 mRNAs was observed.

Given the importance of p450scc and 3βHSD in the steroidogenesis, examination of the expression of the two enzymes in the experimental group remains relatively high. We assume that Leydig cells contain constitutive levels of p450scc mRNAs but show no activity. These results in sheep confirm and demonstrate an age-dependent response of Leydig cells to caloric restriction. In addition, our data suggest that the stimulatory effect of restriction on the expression and enzymatic activity of 3βHSD is influenced by external factors closely related to dietary lipid composition (Rasmussen et al., 2013, Dorgan et al., 1996). Therefore, dietary lipids could modulate *in vivo* the physico-biochemical properties of testicular lipids. On the basis of the immunoblot observations, we showed modulation of the phosphorylation level of p44 / p42 (Erk ½) within the two experimental groups. Inhibition of Erk½ did not lead to a decrease in the expression of StAR, but on the contrary is stabilized at a sufficiently correct level to maintain steroidogenesis in Ouled-Djellal sheep. Thus, it appears that the difference observed in the testicular extracts between the active and non-active form of Erk ½ can be explained by the compartmentalization of the phosphorylated Erk between the cytoplasm or the nucleus where the steroidogenesis is carried out or it exercises its functions of transcriptions (Martinat et al., 2005).

Taken together, we could conclude that AMPK (pAMPK), IL-1α, Nur77 (Nr4a1) and StAR could be involved in the regulation of steroidogenesis in Leydig cells by caloric restriction *in vivo* in the Ouled-Djellal sheep. The p38 MAPK (Svechnikov et al., 2003) could be also a signaling pathway implicated in this regulation.

In summary, the present findings indicate that AMPK and p-AMPK are expressed in ovine testis and regulated by nutritional status (control vs. restricted). Furthermore, nutritional

status also influenced steroidogenesis in ovine testis. In the control group, testosterone production was regulated by StAR protein expression levels via ERK1/2 and AMPK-dependent pathways. Dietary restriction generated an increase in plasma lipoprotein levels; this effect was likely mediated by systemic pro-inflammatory cytokines. However, further studies are needed to confirm or refute these hypotheses.

Acknowledgements

This work is supported by Drylands Research Laboratory grants (Algerian Ministry of high education and scientific: project F 002 2009 0027). We are grateful to Dr. ADEL (Experimental station director) for his assisting in the conduct of experiment, Dr. Z. BOUGUERMOUH (Nutristar International) for his assistance by bringing us the technical and logistical tools required and we also thank Amina KOURI for technical assistance

References

Abdou, H.S., Bergeron, F., Tremblay, J.J., 2014. A cell-autonomous molecular cascade initiated by AMP-activated protein kinase represses steroidogenesis. Molecular and cellular biology 34, 4257-4271.

Anderson, C.M., Mendelson, C.R., 1985. Regulation of steroidogenesis in rat Leydig cells in culture: effect of human chorionic gonadotropin and dibutyryl cyclic AMP on the synthesis of cholesterol side chain cleavage cytochrome P-450 and adrenodoxin. Archives of biochemistry and biophysics 238, 378-387.

Bauman, D.E., Mather, I.H., Wall, R.J., Lock, A.L., 2006. Major advances associated with the biosynthesis of milk. Journal of dairy science 89, 1235-1243.

Choi, S.L., Kim, S.J., Lee, K.T., Kim, J., Mu, J., Birnbaum, M.J., Soo Kim, S., Ha, J., 2001. The regulation of AMP-activated protein kinase by H(2)O(2). Biochemical and biophysical research communications 287, 92-97.

Clark, B.J., Wells, J., King, S.R., Stocco, D.M., 1994. The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR). The Journal of biological chemistry 269, 28314-28322.

Djouder, N., Tuerk, R.D., Suter, M., Salvioni, P., Thali, R.F., Scholz, R., Vaahtomeri, K., Auchli, Y., Rechsteiner, H., Brunisholz, R.A., Viollet, B., Makela, T.P., Wallimann, T., Neumann, D., Krek, W., 2010. PKA phosphorylates and inactivates AMPKalpha to promote efficient lipolysis. The EMBO journal 29, 469-481.

Fernandez, M.L., West, K.L., 2005. Mechanisms by which dietary fatty acids modulate plasma lipids. The Journal of nutrition 135, 2075-2078.

Galardo, M.N., Riera, M.F., Pellizzari, E.H., Cigorraga, S.B., Meroni, S.B., 2007. The AMP-activated protein kinase activator, 5-aminoimidazole-4-carboxamide-1-b-D-ribonucleoside,

regulates lactate production in rat Sertoli cells. Journal of molecular endocrinology 39, 279-288.

Gallet, C., Dupont, J., Campbell, B.K., Monniaux, D., Guillaume, D., Scaramuzzi, R.J., 2011. The infusion of glucose in ewes during the luteal phase increases the number of follicles but reduces oestradiol production and some correlates of metabolic function in the large follicles. Anim Reprod Sci 127, 154-163.

Ham, S., Brown, K.A., Simpson, E.R., Meachem, S.J., 2016. Immunolocalisation of aromatase regulators liver kinase B1, phosphorylated AMP-activated protein kinase and cAMP response element-binding protein-regulated transcription co-activators in the human testis. Reproduction, fertility, and development.

Hardie, D.G., Ashford, M.L., 2014. AMPK: regulating energy balance at the cellular and whole body levels. Physiology (Bethesda) 29, 99-107.

Hardie, D.G., Salt, I.P., Hawley, S.A., Davies, S.P., 1999. AMP-activated protein kinase: an ultrasensitive system for monitoring cellular energy charge. The Biochemical journal 338 (Pt 3), 717-722.

Hochereau-De Reviers, M.T., Copin, M., Seck, M., Monet-Kuntz, C., Cornu, C., Fontaine, I., Perreau, C., Elsen, J.M., Boomarov, 1990. Stimulation of testosterone production by PMSG injection in the ovine male: effect of breed and age and application to males carrying or not carrying the “F” Booroola gene. Animal Reproduction Science 23, 21-32.

Hurtado de Llera, A., Martin-Hidalgo, D., Gil, M.C., Garcia-Marin, L.J., Bragado, M.J., 2015. AMPK up-activation reduces motility and regulates other functions of boar spermatozoa. Molecular human reproduction 21, 31-45.

Hurtado de Llera, A., Martin-Hidalgo, D., Gil, M.C., Garcia-Marin, L.J., Bragado, M.J., 2016. New insights into transduction pathways that regulate boar sperm function. Theriogenology 85, 12-20.

Illingworth, B., Larner, J., Cori, G.T., 1952. Structure of glycogens and amylopectins. I. Enzymatic determination of chain length. The Journal of biological chemistry 199, 631-640.

Kahn, B.B., Alquier, T., Carling, D., Hardie, D.G., 2005. AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. Cell metabolism 1, 15-25.

Kanoh, Y., Bandyopadhyay, G., Sajan, M.P., Standaert, M.L., Farese, R.V., 2001. Rosiglitazone, insulin treatment, and fasting correct defective activation of protein kinase C-zeta/lambda by insulin in vastus lateralis muscles and adipocytes of diabetic rats. Endocrinology 142, 1595-1605.

McBride, A., Ghilagaber, S., Nikolaev, A., Hardie, D.G., 2009. The glycogen-binding domain on the AMPK beta subunit allows the kinase to act as a glycogen sensor. Cell metabolism 9, 23-34.

McNamara, D.J., 1992. Dietary fatty acids, lipoproteins, and cardiovascular disease. Advances in food and nutrition research 36, 253-351.

Melendez-Hevia, E., Waddell, T.G., Shelton, E.D., 1993. Optimization of molecular design in the evolution of metabolism: the glycogen molecule. The Biochemical journal 295 (Pt 2), 477-483.

Nguyen, T.M., Alves, S., Grasseau, I., Metayer-Coustard, S., Praud, C., Froment, P., Blesbois, E., 2014. Central role of 5'-AMP-activated protein kinase in chicken sperm functions. Biology of reproduction 91, 121.

Rasmussen, M.K., Ekstrand, B., Zamaratskaia, G., 2013. Regulation of 3beta-hydroxysteroid dehydrogenase/Delta(5)-Delta(4) isomerase: a review. International journal of molecular sciences 14, 17926-17942.

Reshef, L., Olswang, Y., Cassuto, H., Blum, B., Croniger, C.M., Kalhan, S.C., Tilghman, S.M., Hanson, R.W., 2003. Glyceroneogenesis and the triglyceride/fatty acid cycle. The Journal of biological chemistry 278, 30413-30416.

Salt, I.P., Johnson, G., Ashcroft, S.J., Hardie, D.G., 1998. AMP-activated protein kinase is activated by low glucose in cell lines derived from pancreatic beta cells, and may regulate insulin release. The Biochemical journal 335 (Pt 3), 533-539.

Scaramuzzi, R.J., Zouaidi, N., Menassol, J.B., Dupont, J., 2015. The effects of intravenous, glucose versus saline on ovarian follicles and their levels of some mediators of insulin signalling. Reproductive biology and endocrinology : RB&E 13, 6.

Shearer, J., Graham, T.E., 2002. New perspectives on the storage and organization of muscle glycogen. Canadian journal of applied physiology = Revue canadienne de physiologie appliquee 27, 179-203.

Shoba, B., Lwin, Z.M., Ling, L.S., Bay, B.H., Yip, G.W., Kumar, S.D., 2009. Function of sirtuins in biological tissues. Anat Rec (Hoboken) 292, 536-543.

Simpson, E.R., Waterman, M.R., 1988. Regulation of the synthesis of steroidogenic enzymes in adrenal cortical cells by ACTH. Annual review of physiology 50, 427-440.

Suter, M., Riek, U., Tuerk, R., Schlattner, U., Wallimann, T., Neumann, D., 2006. Dissecting the role of 5'-AMP for allosteric stimulation, activation, and deactivation of AMP-activated protein kinase. The Journal of biological chemistry 281, 32207-32216.

Tartarin, P., Guibert, E., Toure, A., Ouiste, C., Leclerc, J., Sanz, N., Briere, S., Dacheux, J.L., Delaleu, B., McNeilly, J.R., McNeilly, A.S., Brillard, J.P., Dupont, J., Foretz, M., Viollet, B., Froment, P., 2012. Inactivation of AMPKalpha1 induces asthenozoospermia and alters spermatozoa morphology. Endocrinology 153, 3468-3481.

To, K., Yamaza, H., Komatsu, T., Hayashida, T., Hayashi, H., Toyama, H., Chiba, T., Higami, Y., Shimokawa, I., 2007. Down-regulation of AMP-activated protein kinase by calorie restriction in rat liver. Experimental gerontology 42, 1063-1071.

Tosca, L., Froment, P., Solnais, P., Ferre, P., Foufelle, F., Dupont, J., 2005. Adenosine 5'-monophosphate-activated protein kinase regulates progesterone secretion in rat granulosa cells. Endocrinology 146, 4500-4513.

Turley, M.L., Skeaff, C.M., Mann, J.I., Cox, B., 1998. The effect of a low-fat, high-carbohydrate diet on serum high density lipoprotein cholesterol and triglyceride. European journal of clinical nutrition 52, 728-732.

Viollet, B., Andreelli, F., Jorgensen, S.B., Perrin, C., Flamez, D., Mu, J., Wojtaszewski, J.F., Schuit, F.C., Birnbaum, M., Richter, E., Burcelin, R., Vaulont, S., 2003a. Physiological role of AMP-activated protein kinase (AMPK): insights from knockout mouse models. Biochemical Society transactions 31, 216-219.

Viollet, B., Andreelli, F., Jorgensen, S.B., Perrin, C., Geloen, A., Flamez, D., Mu, J., Lenzner, C., Baud, O., Bennoun, M., Gomas, E., Nicolas, G., Wojtaszewski, J.F., Kahn, A., Carling, D., Schuit, F.C., Birnbaum, M.J., Richter, E.A., Burcelin, R., Vaulont, S., 2003b. The AMP-activated protein kinase alpha2 catalytic subunit controls whole-body insulin sensitivity. The Journal of clinical investigation 111, 91-98.

Wojtaszewski, J.F., Jorgensen, S.B., Hellsten, Y., Hardie, D.G., Richter, E.A., 2002. Glycogen-dependent effects of 5-aminoimidazole-4-carboxamide (AICA)-riboside on AMP-activated protein kinase and glycogen synthase activities in rat skeletal muscle. Diabetes 51, 284-292.

Yoneda, M., Guo, Y., Ono, H., Nakatsu, Y., Zhang, J., Cui, X., Iwashita, M., Kumamoto, S., Tsuchiya, Y., Sakoda, H., Fujishiro, M., Kushiyama, A., Koketsu, Y., Kikuchi, T., Kamata, H., Nishimura, F., Asano, T., 2010. Decreased SIRT1 expression and LKB1 phosphorylation

occur with long-term high-fat diet feeding, in addition to AMPK phosphorylation impairment in the early phase. Obesity research & clinical practice 4, e163-246.

Zenimaru, Y., Takahashi, S., Takahashi, M., Yamada, K., Iwasaki, T., Hattori, H., Imagawa, M., Ueno, M., Suzuki, J., Miyamori, I., 2008. Glucose deprivation accelerates VLDL receptor-mediated TG-rich lipoprotein uptake by AMPK activation in skeletal muscle cells. Biochemical and biophysical research communications 368, 716-722.

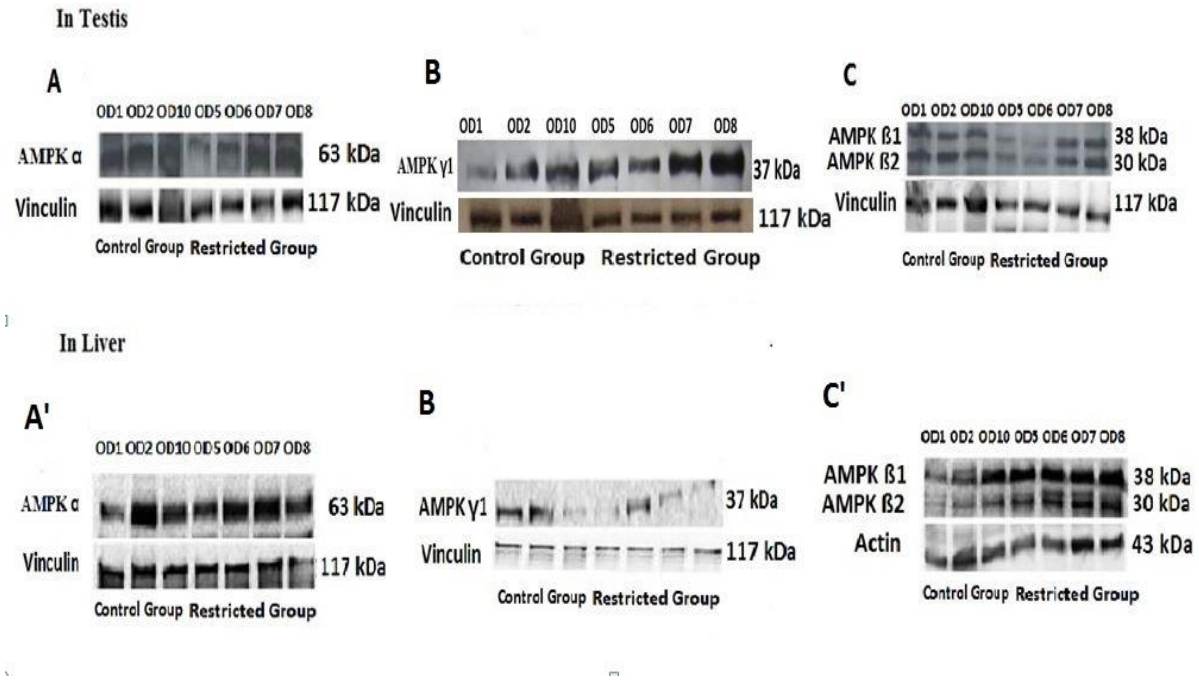


Fig.1. AMPK subunits expression in metabolic and reproductive tissues in both Control (OD1, OD2, OD10) and Restricted (OD5, OD6, OD7, OD8) Groups

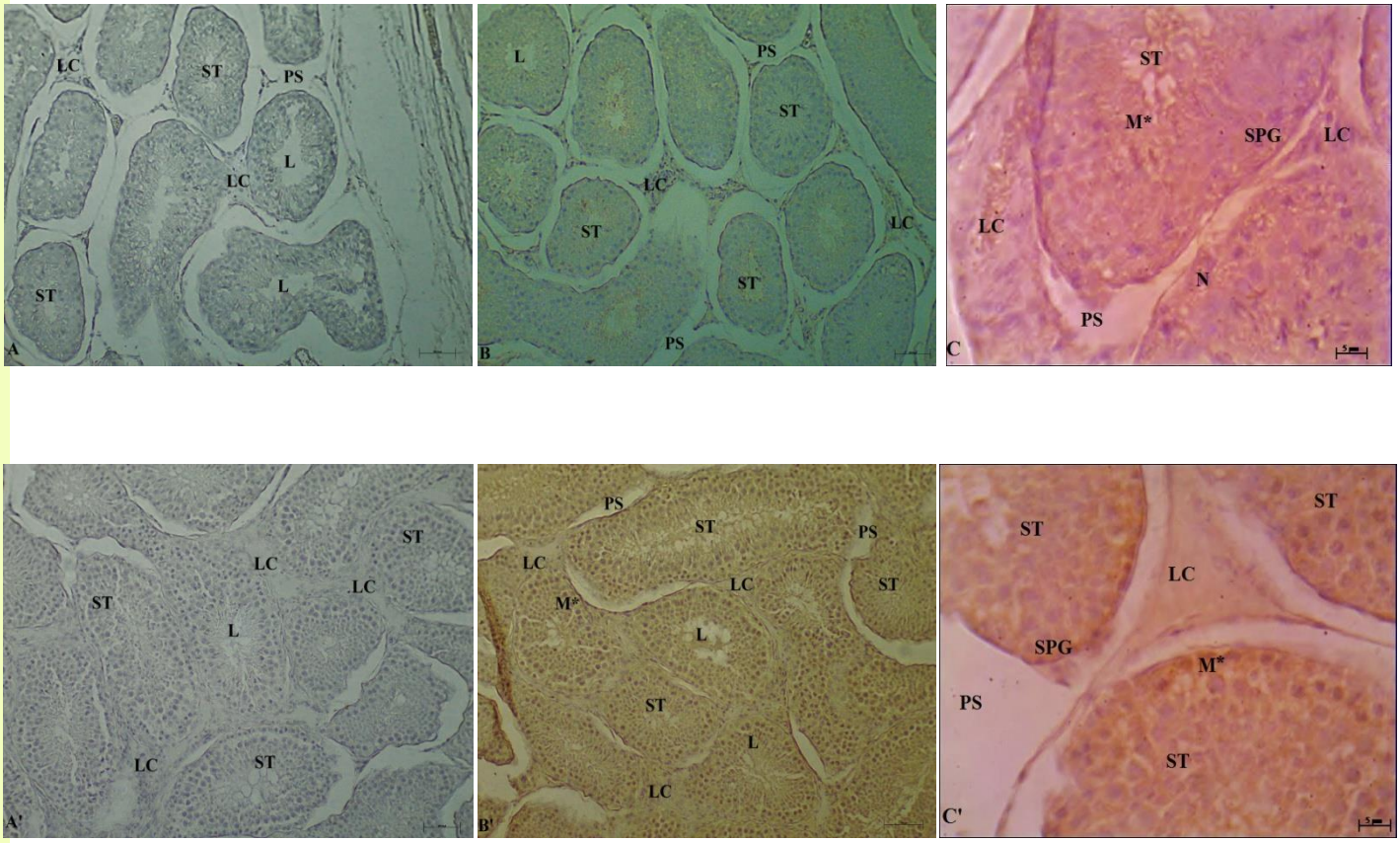


Fig. 2. Immunohistochemical analysis of paraffin-embedded sheep testis, showing cytoplasmic localisation of Phospho-AMPKα (Thr172)(40H9) Rabbit mAb (Fig. 2B, 2B', Gx10 , 2C, 2C', Gx40) in the presence of negative control (Fig. 2A, 2A', Gx10). Phospho-AMPKα is detected in all testicular structures; Leydig cells (LC), Spermatogonia (SPG), Spermatocytes (SPY), Spermatids (SPT), Spermatozoa (SPZ) and Sertoli cells (SC). Phosphorylated AMPK in testis of animals fed with 1000 grams of concentrate per day (Fig. 2A, 2B, 2C) and those fed with 400 grams per day (Fig. 2A', 2B', 2C') show a relative abondance of immunostaining in the two groups.

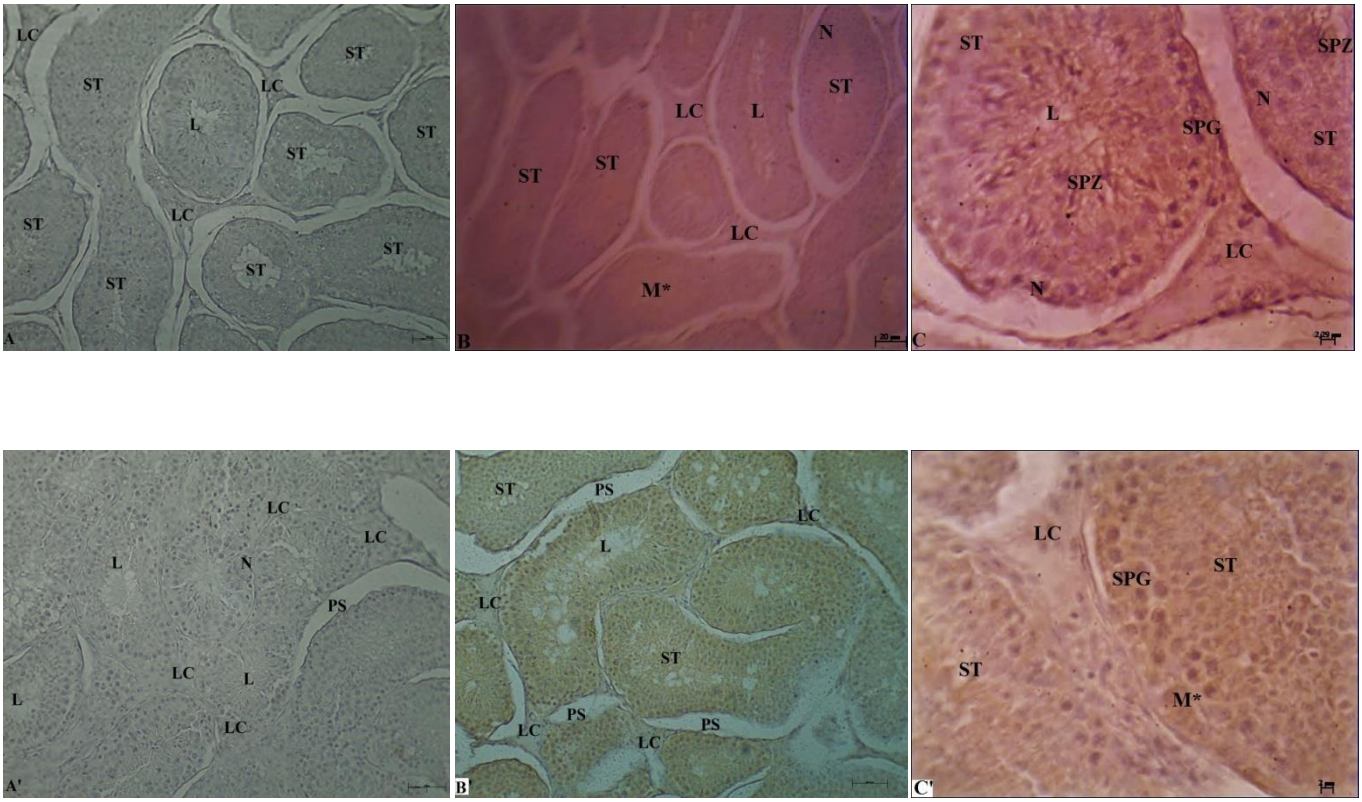


Fig. 3. Immunohistochemical analysis of paraffin-embedded sheep testis, showing cytoplasmic localisation of AMPKα Rabbit polyclonal antibody (Fig. 3B, 3B', Gx10, 3C, 3C', Gx40) in the presence of negative control (Fig. 3A', 3A', Gx10). AMPKα is detected in all testicular structures; Leydig cells (LC), Spermatogonia (SPG), Spermatocytes (SPY), Spermatids (SPT), Spermatozoa (SPZ) and Sertoli cells (SC). Fig. 3B and 3C represent figures of animals fed with 1000 grams of concentrate per day and those (Fig. 3B', 3C') fed with 400 grams per day.

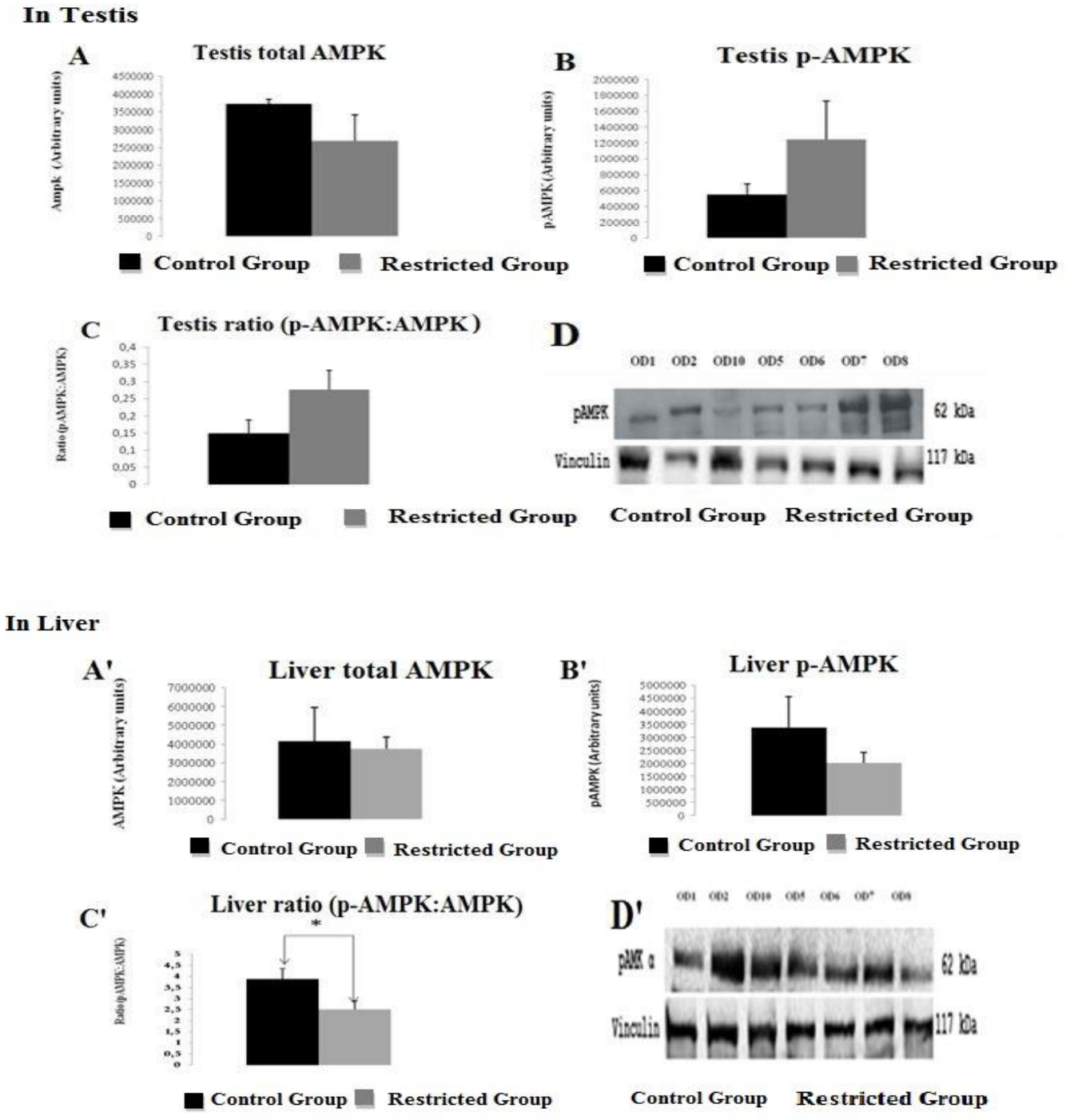


Fig. 4. Effect of control (n=3) and restricted (n=4) fed phases on AMPK activity in testis and liver tissues. Representative Western blots results for total AMPK (A, A'), Phospho-Thr 172 AMPK α (B, B'/D, D') and the abundance of p-AMPK α (C, C') in Ouled-Djellal sheep. Data represent the means ± SEM.

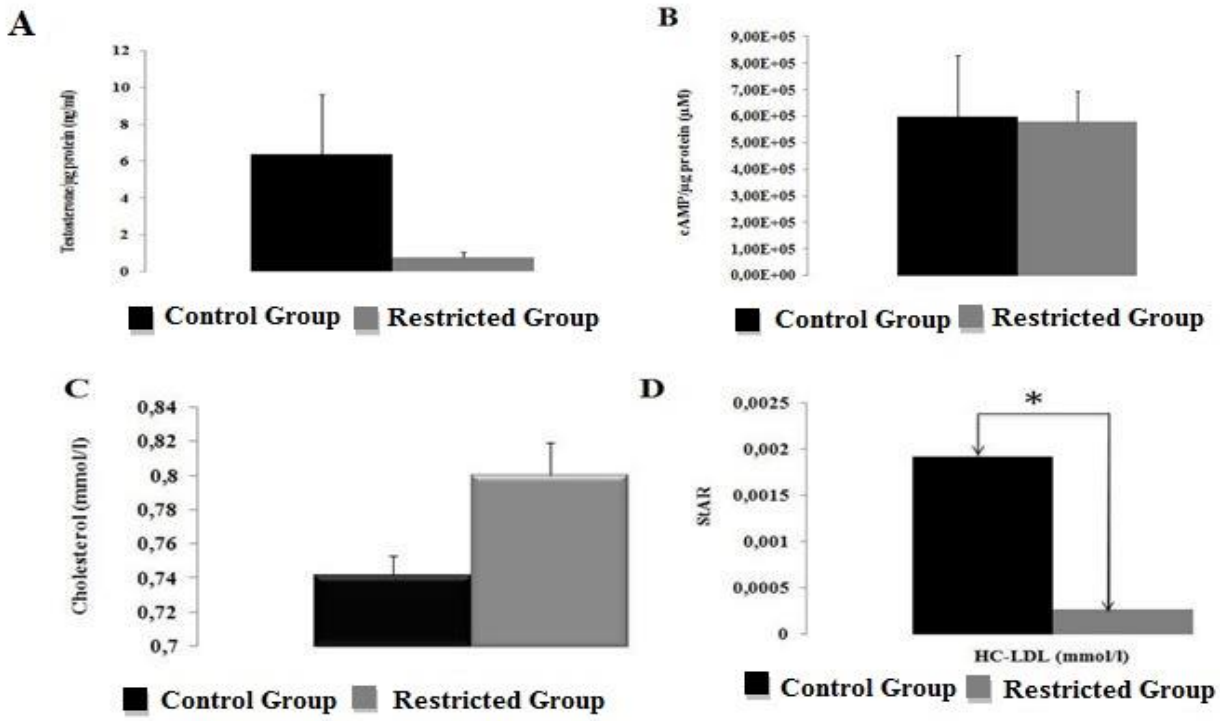


Fig. 5. Estimated of intra-testicular in testosterone (A), cAMP (B), cholesterol (C) levels and validation of reciprocity between the StAR protein and its potential substrate (D)

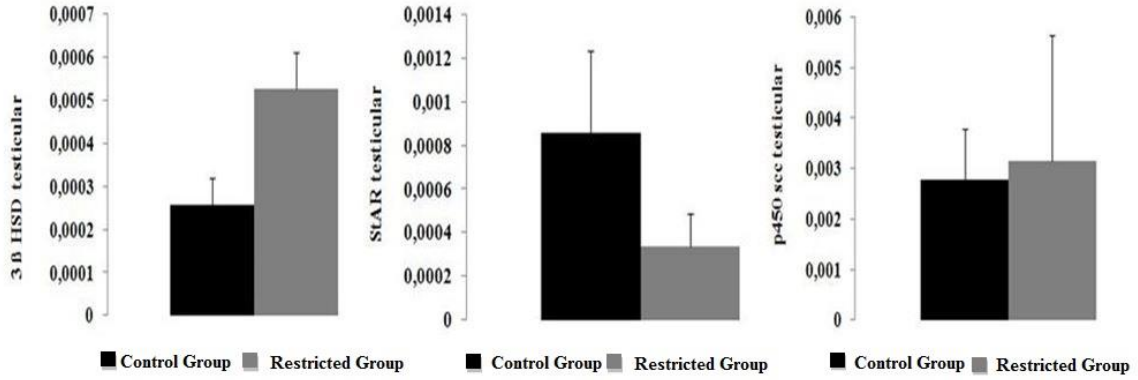


Fig. 6. Expression of testicular steroidogenic enzymes quantified by RTq-PCR

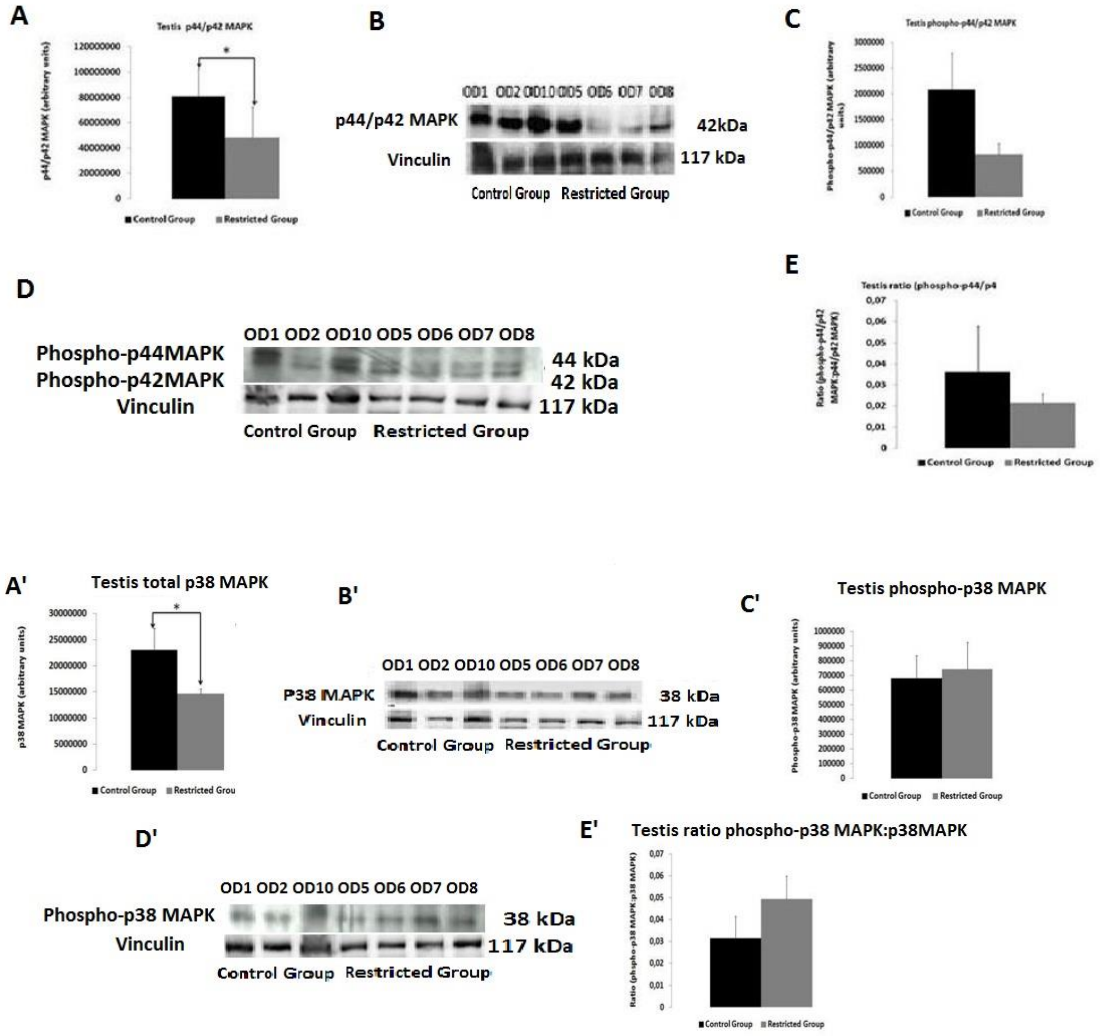


Fig. 7. Effect of two nutitional levels [(Control Group (OD1, OD2, OD10) n=3; Diet1: 1000g/day/animal/Restricted Group (OD5,OD6,OD7,OD8), n=4; Diet2: 400 g/day/animal)] on testitular expression of p44/42 MAPK, Phospho-p44/42 MAPK, , p38 MAPK and Phospho-p38 MAPK. In native gel electrophoresis and immunoblotting, the extracted cellular proteins from testis were analyzed using specific antibodies. Relative abundance of total (Fig. 7A and 7B) and Phosphorylated forms of p44/42 MAPK (Fig. 7C and 7D) and p38 MAPK (Fig.7A', 7B', 7C', 7D' and 7E'),) in according to the quantitative distribution of concentrate. Proportion of phosphorylated to total p44/42 MAPK (Fig. 7E) and p38 MAPK (Fig. 7E'), were also evaluated (relative abundance of protein normalized to Vinculin). All values are group means ± ESM and Asterix indicates statistical significance ($p < 0.05$).

Table 1: Primer sequences for specific oligonucleotide primer pairs (F= forward, R= Reverse), expected amplicon size (bp) and accession number.

Abbreviation	Gene name	Function	Oligonucleotides (5'-3')	Amplicon (bp)	Accession number
CYCLOA	Cyclophilin A	Cyclosporin binding protein	F:TCCGGGATTTATGTGCCAGGG R:ATCTCCTGTCTTACCACTCAG	206	XM_015095354.1
SDHA	Succinate dehydrogenase flavoprotein subunit A	Electron transporter in the TCA cycle and respiratory chain	F: TATATGGCGCTGGCTGTCTC R:CCTCTTCCCTCGCGGATTTC	168	XM_012097183.1
VUXT	Ubiquitously expressed transcript	Transcriptional activation	F: CATTGAGCGACTCCAGGAAG R:GGCCACATAGATCCGTGAAG	112	XM_004022128.3
3betaHSD	3-beta-hydroxysteroid dehydrogenase	A crucial role in the biosynthesis of hormonal steroids	F: TGGATGAGCAGTGCCTGAAG R: AGCTGGGTACCTTTCACATT	130	FJ007375
StAR	Steroidogenic acute regulatory protein	Cholesterol carrier	F: TCTCCTAGGTTCTCAGCTGG R: GGATCACTTTACTCAGCACC	169	NM 001009243
P450scc	Cholesterol side-chain cleavage enzyme	Mitochondrial enzyme that catalyzes conversion of cholesterol to pregnenolone	F: TCAGCCCTGGTCAAAGCCTG R:CGAGGGGTCCTCGTGGAGAT	120	S65754

Table 1

Chronic diet effects on both anthropometric characteristics and biochemical parameters in Ouled-Djellal sheep.

	Item	*Control-Fed-Phase Group*	*Restricted-Fed-Phase Group*
Anthropometric characteristics	*Body weight (Kg)*	*37.17 ± 1,15*	*26.12 ± 0,085*
	Height at the withers (cm)	*69.93 ± 0,58*	*66.18 ± 0,38*
	Thoracic perimeter (cm)	*84.79 ± 0.84*	*80.83 ± 0.45*
	Scrotal circumference (cm)	*24.30 ± 0.57*	*18.91 ± 0.48*
Biochemical parameters	*Glucose (mmol/l)*	*4.34 ± 0,08*	*3.70 ± 0,06*
	Total protein (g/dl)	*6.11 ± 0,09*	*6.18 ± 0,10*
	Triglyceride (mmol/l)	***0.25 ± 0.015 vs***	***0.28 ± 0.016 *****
	Cholesterol (mmol/l)	*1.17 ± 0.033*	*1.70 ± 0.057*
	HC-HDL (mmol/l)	*0.69 ± 0.02*	*0.82 ± 0.02*
	HC-LDL (mmol/l)	*0.54 ± 0.03*	*0.71 ± 0.04*
	HC-VLDL (mmol/l)	***0.11 ±0.007 vs***	***0.13 ± 0.007 *****
	Total fat (mmol/l)	*3.69 ± 0.09*	*4.54 ± 0.15*
	NEFAs (mM/l)	*307.71 ± 97.45*	*257.40 ± 22.45*

All data are expressed as mean ± SEM.
Only triglycerides and the HC-VLDL lipoprotein fraction showed a statistically difference between the two groups for nutritional phases proposed *P<0.05.