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Effects of Mefepronic Acid (2-Phenoxy–2-Methyl Propionic Acid) on Hepatic Metabolism and Reproductive Parameters in Postpartum Dairy Cows

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Abstract: This study investigates the effects of mefepronic acid (MA), a PPAR-α agonist, on hepatic metabolic functions and reproduction of postpartum dairy cows. Sixty Friesian cows were divided into Group A (administered 5g of MA IM, within 24 hrs after calving, on the 3rd and 5th day postpartum) and Group B (control). All the cows were blood sampled within 24 hrs of calving (Day 0), on Day 3, 5, 10, 15, 30, and 40 postpartum. On plasma, metabolic and biochemical parameters were determined. Liver biopsies were performed on Day 0, 15 and 30 for the evaluation of hepatic lipid and glycogen content. Reproductive parameters were also evaluated.

In Group A, blood HDL, glucose and cholesterol increased till the end of the study, in accordance with the histological results. PPAR-α immunopositive cells increased in liver slices of Group A, too. Reproductive parameters improved in Group A. This study highlights the beneficial effects of mefepronic acid on the hepatic metabolism and reproductive parameters of post-partum dairy cows.

Keywords: Dairy cow, mefepronic acid, liver metabolism, reproduction.

INTRODUCTION

The transition period of the cow is the period ranging from the last three weeks of pregnancy until the first three weeks after calving [1, 2]. During this period the cow undergoes major physiological changes due to the onset of lactation and the consequent high metabolic requirements of the udder [3, 4]. These changes lead to an extensive peripheral mobilization of long-chain fatty acids from the adipose tissue [5]. These substances represent the main energy source for the cow in this period and reach the bloodstream as non-esterified fatty acids (NEFA) [4]. Once in the liver, NEFA may undergo three different metabolic processes: complete oxidation, partial oxidation leading to the formation of ketone bodies or re-esterification to triglycerides [6, 7].

In healthy cows, a mild lipid mobilization is considered physiological, but when the energetic deficit is too high, excessive lipids are mobilized and the liver cannot fully metabolize them. This condition leads to the accumulation of triglycerides in the liver and, consequently, to the development of hepatic steatosis or "fatty liver" [8] and other related pathologies such as ketosis and displaced abomasum [9, 10].

Lipid metabolism and adipocyte differentiation are regulated by several genes whose expression is modulated by the activation of the peroxisome proliferator-activated receptors (PPARs), belonging to the superfamily of nuclear receptors [11]. There are three PPAR isoforms, alpha, beta/delta and gamma, the former of which is abundantly expressed in those tissues presenting high lipid catabolic activity, such as liver, kidneys, heart, skeletal muscle and brown adipose tissue [12]. PPARα is activated by fatty acids, prostaglandins and fibrates, *i.e*. drugs used in human medicine for their hypolipidaemic action [13]. Besides, PPAR α activation by its agonists has been shown to down-regulate genes involved in the immune response and inflammation [14, 15].

As to metabolic effects, 2-phenoxy–2-methyl propionic acid, or mefepronic acid (MA) stimulates the physiological activities of the liver and the activation of digestive processes [16]. MA is marketed as Hepagen® (Fatro, Bologna, Italy) and used for treating ketosis, liver diseases, and fat cow syndrome in veterinary practice.

Given these premises and given the tight relationship between metabolism and reproductive activity [2, 5] this study aims at investigating the effect of MA on metabolic and reproductive parameters, from a clinical and histological point of view. Moreover, since it was suggested that MA might act on PPARα, the presence of its effect on the expression of PPARα in the hepatocytes was evaluated, too.

MATERIAL AND METHODS

All the procedures were carried out in accordance with the Italian Legislation on animal care (D.L.vo 116/92) and following the written consensus of the animal owner.

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Animals

60 post-partum Friesian cows, aged 5 to 8 years, calved in March and April 2013, bred in two commercial dairy farms in the South of Italy, with a consistency of 150-200 cows each, were enrolled in this study. The animals had a mean body weight of 600 kg (range 560 to 650 kg) and an average milk production of 8,300 to 8,500 kg per lactation. The cows were housed in tie stalls and fed hay, concentrate, and minerals, with access to fresh drinkable water *ad libitum*. Both groups underwent a thorough clinical exam (including rectal palpation and ultrasonography), in order to exclude any pathological condition. Moreover, all animals were diagnosed free from common parasites and declared officially free from bovine diarrhoea, brucellosis, bovine leukosis virus and tuberculosis. Body Condition Score (BCS) was assessed by the same experienced evaluator and was esteemed 3.5±0.3 (in a scale from 1 to 5).

The subjects were randomly divided into 2 groups (homogeneous for mean age, body weight, BCS and milk production): Group MA (n = 30 cows), administered 50 mL/per head of Hepagen® (Fatro S.p.A.) (corresponding to 5 g of MA) IM, within 24 hrs *post partum* and at three and five days *post partum*; Group CTL (n = 30 cows) given 50 mL/per head of sterile saline solution (NaCl 0.9%) IM, at the same time-points as Group MA.

The cows were followed (clinical exam and BCS) throughout the experimental period (from calving to 40 days *post partum*), in order to detect any occurring disease and disorder, drug treatment and culling causes.

Blood Analyses

Blood samples were collected at the following times: 24 hrs after calving (D0), 3 (D3), 5 (D5), 10 (D10), 15 (D15), 30 (D30) and 40 (D40) days after calving. Blood collections on D0, 3, 5 were performed just prior to treatment (Hepagen or saline solution). All blood samples were collected by coccygeal venipuncture into vacutainer tubes (Lithium Heparin) maintained at 4°C. Once in the lab, blood was centrifuged at 1620 *xg* at 4°C, within 1 hour. Plasma was stored in Eppendorf tubes at -20°C until analytical determination.

The following parameters were evaluated: glucose (GLU) (UV enzymatic, linearity: 0.11-41.6 mmol/L; sensitivity: 0.11 mmol/L), non esterified fatty acids (NEFA) (colorimetric, linearity of reaction: 0-2 mEq/L), beta-hydroxybutyric acid (BHBA) (colorimetric, linearity of reaction: 0.1-3.2 mmol/L, cholesterol (CHOL) (enzymatic colorimetric, linearity of reaction: 0.08-20.7 mmol/L, analytic sensitivity: 0.08 mmol/L), triglycerides (TG), aspartate aminotransferase (AST) (UV by IFCC, linearity: 4-800 U/L, analytic sensitivity: 4 U/L), alanine aminotransferase (ALT) (UV by IFCC, linearity: 4-600 U/L, analytic sensitivity: 4 U/L), gamma-glutamyltransferase (GGT) (enzymatic colorimetric, linearity: 3-1200 U/L, analytic sensitivity: 3 U/L), serum alkaline phosphatase (SAP) (colorimetric by IFCC, linearity: 1-1200 U/L, analytic sensitivity: 0.67 U/L), total proteins (TP) (colorimetric, biuret, linearity: 2-150 g/L, analytic sensitivity: 0.08 µmol/L) albumin (ALB) (colorimetric, linearity: 10-70 g/L, analytic sensitivity: 2 g/L), total, direct and indirect bilirubin (TBil, DBil and IBil, respectively) (T Bil: liquid with ion of diazone, linearity: 1.71-171 mmol/L, analytic sensitivity: 1.71 mmol/L; DBil: Jendrassen method, linearity: 0.2-.50 mmol/L, analytic sensitivity: 0.2 mmol/L) and high density lipoprotein (HDL) (linearity: 0.08-3.1 µmol/L, analytic sensitivity: 0.1 mmol/L. All the above mentioned parameters were tested with an analyzer for clinic biochemistry, Hitachi 911 (Roche Diagnostics).

Liver Biopsy and Analysis

Liver biopsies were performed on Day 0, 15, 30 on 10 cows of Group MA and CTL, respectively, randomly selected. On each cow, the first liver biopsy (D0) was performed soon after blood sampling and just before the administration of MA. A slight sedation with xilazine (Rompun®, Bayer, Milan, Italy - 0.03 mg/kg) was used and lidocaine (5 mL) (Lidocaine 2%®, Fort Dodge, Bologna, Italy) was locally infiltrated around the location of needle introduction. Biopsies were performed at the 10th right intercostal space using a 15 gauge Meneghini modified bioptic needle (15 cm long), following the technique described by [17], and the liver sample obtained was immediately put in a Petri dish for dividing the samples into two aliquots: the former was placed in a vial containing glutaraldehyde 4% buffered with cacodylate 0.1 M at pH 7.2, the latter was placed in a vial containing formalin 10% (v/v) buffered with 0.1 M PBS pH 7.4.

The liver samples underwent histological and immune-histochemical studies to evaluate morphology, lipid and glycogen content and PPARα expression.

Lipid and Glycogen Content

As to the evaluation of lipid content, samples were fixed at 4°C for 4 hrs in glutaraldehyde 4% buffered with cacodylate 0.1 M at pH 7.2, post-fixed in 1% osmium tetroxide for 2 hrs at 4°C, dehydrated in increasing concentrations of ethanol (30%, 50%, 70%, 80%, 95%), infiltrated with propylene oxide and embedded in Epon-Araldite. Two-micrometer semi-thin sections cut with an ultra-microtome were coloured with 1% toluidine blue. Five fields at 100X were used to assess the percentage of parenchyma occupied by lipid droplets; the diameter of the larger lipid droplets was estimated with an image analyser (Quantimet 500/W, Leika) connected to a digital camera.

Histological features and glycogen content were evaluated on samples fixed at 4° C for 24 hrs in formalin 10% (v/v) buffered with 0.1 M PBS pH 7.4, dehydrated in increasing concentrations of ethanol (50%, 70%, 80%, 95%, 100%), clarified with xylol and embedded in paraffin wax (fusion temperature 56°-58 °C). Five- micrometer thick sections were cut with an ultra-microtome and stained with haematoxylin-eosin for the general histological evaluation and with haematoxylin-PAS for estimating glycogen content.

Immuno-Histochemical Detection of PPARα

De-waxed and re-hydrated liver sections were pre-treated with two microwave cycles (2 x 5 min at 450 W in 0.01 M citrate buffer, pH 6.0) to unmask the antigen. Then the

sections were incubated for 30 min in a solution of 0.3% H_2O_2 in methanol to inhibit endogenous peroxidase activity and were rinsed with PBS-1% BSA. Non-specific binding sites for immunoglobulins were blocked by incubation in 5% Normal Goat Serum (NGS) in PBS-BSA for 30 min. Then, the sections were incubated for 17 hrs at 4°C in a moist chamber with a 1:50 dilution of primary goat polyclonal antibody against human PPARα (C-20) (Santa Cruz Biotechonogy, Inc., Heidelberg, Germany).

The sections were then incubated for 30 min with diluted biotinylated rabbit anti-goat IgG. After washing for 15 min in PBS-BSA, immunohistochemical visualisation was obtained using a Vecta-lab "Elite" (ABC) kit (Vector Laboratories, Peterborough, UK). Peroxidase activity was visualised by incubation with both 0.01% H_2O_2 and 0.05% diaminobenzidine-tetrahydrochloride (Sigma-Aldrich, Milan, Italy) in 0.05 M Tris buffer, pH 7.2, for 5 min to reveal the brown immuno-reactive cells. To confirm the specificity of the immunoreaction, the following control procedures were performed: 1) replacement of primary antibody with NGS and 2) omission of the primary antibody incubation step.

To determine the number of immunopositive cells, 6 randomly-chosen fields for each biopsy were observed at 40x magnification using a light microscope (Leitz DMRBE) connected to a digital camera (Sony DC 300) and recorded by the image analyser Quantimet 500/W (Leica, UK). Each field measured 60000 μm^2. The data (means ± s.e.) were analyzed as for lipid droplets.

Table 1. Plasma levels (mean ± s.e.) of ALB, SAP, ALT, AST, GGT, DBil, IBil, TBil, BHBA, CHOL, GLU, HDL, TP, TG and NEFA, in Group MA (treatment) and Group CTL (control) at 3 (D3), 5 (D5) and 10 (D10) days after calving. A,B: P<0.01; C,D: P<0.05.

	D3		D5		D10	
	MA	**CTL**	**MA**	**CTL**	**MA**	**CTL**
ALB (g/L)	33.56±0.7	31.67±0.8	33.11±0.76	30.86±0.93	33.22±0.79	29.11±0.8
SAP (U/L)	50.67±2.49	45.78±2.56	58.22±2.4	49.43±3.09	43.78±2.62	38.11±2.5
ALT (U/L)	18.11±0.86	20.33±0.8	17.88±0.75	19.71±1.11	17.11±0.64	17.44±0.75
AST (U/L)	119.56±4.55	101.14±5.5	119.33±5.8	93.67±6.12	110±4.98	96.87±5.45
GGT (U/L)	20.5±1.2	13.62±1.18	22±1.15	12.83±1.32	21.67±1.15	17.56±1.12
DBil (mmol/L)	3.23±0.16	2.33±0.14	2.98±0.12	2.89±0.18	2.67±0.17	2.02±0.14
IBil (mmol/L)	8.83±0.3	7.12±0.35	9.49±0.33	7.15±0.45	6.72±0.29	6.96±0.33
TBil (mmol/L)	12.07±0.49	9.45±0.54	12.48±0.49	10.05±0.89	9.33±0.5	8.97±0.48
BHBA (mmol/L)	0.52±0.03	0.52±0.02	0.6±0.03	0.47±0.03	0.49±0.02	0.65±0.02
CHOL (mmol/L)	1.96±0.05	1.85±0.05	2.01±0.06	1.81±0.07	2.27±0.05	1.94±0.05
GLU (mmol/L)	2.99±0.12 [A]	4.42±0.1 [B]	2.97±0.14 [A]	4.18±0.13 [B]	3.21±0.15	3.92±0.09
HDL (mmol/L)	1.73±0.05	1.7±0.03	1.74±0.06	1.67±0.03	1.93±0.04	1.87±0.03
TP (g/L)	71.11±1.12	70.78±1.18	73.78±1.08	73.42±1.38	79.11±1.1 [A]	69.56±1.21 [B]
TG (mmol/L)	0.15±0.01	0.15±0.01	0.13±0.01	0.14±0.02	0.17±0.01	0.13±0.02
NEFA (mEq/L)	0.62±0.02	0.6±0.02	0.45±0.03	0.55±0.04	0.56±0.02 [C]	0.35±0.03 [D]

Reproductive Parameters

Calving-first oestrus interval, calving-conception interval and pregnancy rate at the first and second Artificial Insemination (A.I.). were considered as reproductive parameters. Typical oestrus signs (vocalisation, oedema of the vulva, mucous vaginal discharge, standing to be mounted) and the detection of a preovulatory follicle on rectal palpation and ultrasonography (SonoSite, MicroMaxx Bothell, WA, USA) performed with a linear multifrequency probe set at 7.5 MHz, let diagnose oestrus Oestrus was detected observing.

Transrectal palpation and ultrasonography on the 40th day after A.I. were used to diagnose pregnancy. 11 (T11) and 13 (T13) days after A.I., blood samples were collected and sera were stored as previously described. Retrospectively, plasma progesterone (P4) concentrations were measured with Progesterone EIA Well [RADIM S.p.A., Pomezia (RM), Italy], on the sera obtained at T11 and T13 after the artificial insemination leading to pregnancy (cross-reactivities: P4: 100%; estradiol <1×10−2%; testosterone <1×10−2%; cortisol <1×10−3%; cholesterol <1×10−3%. The intra-assay and inter-assay precision had coefficients of variation of 2.9% and 4.8%, respectively).

Statistical Analysis

The values were expressed as least squares mean ± s.e. for all the parameters considered.

Table 2. Plasma levels (mean ± s.e.) of ALB, SAP, ALT, AST, GGT, DBil, IBil, TBil, BHBA, CHOL, GLU, HDL, TP, TG and NEFA, in Group MA (treatment) and Group CTL (control) at 10 (D10), 15 (D15) and 30 (D30) days after calving. A,B: P<0.001; C,D: P<0.05; E,F: P<0.05.

	D10		D15		D30		D40	
	MA	**CTL**	**MA**	**CTL**	**MA**	**CTL**	**MA**	**CTL**
ALB (g/L)	33.22±0.79	29.11±0.8	31.89±0.89	29.25±1.1	34.22±0.79	32.5±1.1	34.89±0.8	29.87±1.02
SAP (U/L)	43.78±2.62	38.11±2.5	41.44±1.89	36.62±2	41.22±2.03	41.12±1.89	41.78±1.9	42.87±2.2
ALT (U/L)	17.11±0.64^{A}	17.44±0.75	16.55±0.8^{A}	16±0.9	22.2±0.9	20.12±0.7	25.55±0.9^{B}	21.87±1
AST (U/L)	110±4.98	96.87±5.45	91.78±4.06	96.62±4.3	92.22±4.13	87.75±3.9	92.78±3.9	86.62±4.2
GGT (U/L)	21.67±1.15	17.56±1.12	18.56±1.12	17±1.15	19.89±1.1	17.87±1.12	21.55±1.1	18.12±1.15
DBil (mmol/L)	2.67±0.17	2.02±0.14	2.47±0.15	1.79±0.14	2.47±0.14	1.86±0.12	1.86±0.18	1.64±0.19
IBil (mmol/L)	6.72±0.29	6.96±0.33	6.27±0.22	6.86±0.2	5.91±0.19	5.76±0.25	5.91±0.23	5.07±0.19
TBil (mmol/L)	9.33±0.5	8.97±0.48	8.74±0.48	8.65±0.4	8.37±0.3	7.62±0.39	7.78±0.46	6.71±0.48
BHBA (mmol/L)	0.49±0.02	0.65±0.02	0.49±0.03	0.54±0.04	0.45±0.03	0.65±0.04	0.48±0.02	0.55±0.03
CHOL (mmol/L)	2.27±0.05^{AC}	1.94±0.05^{AC}	2.52±0.08^{AC}	2.20±0.12^{A}	3.66±0.09^{D}	3.22±0.12^{D}	4.34±0.07^{B}	3.76±0.13^{B}
GLU (mmol/L)	3.21±0.15	3.92±0.09	3.22±0.08	3.72±0.09	3.89±0.09	3.81±0.08	3.5±0.08	3.84±0.09
HDL (mmol/L)	1.93±0.04^{AC}	1.87±0.03^{AC}	2.17±0.05^{A}	2.07±0.08^{A}	2.99±0.06^{D}	2.9±0.04^{D}	3.53±0.03^{B}	3.29±0.05^{B}
TP (g/L)	79.11±1.1	69.56±1.21	81.22±1.9	72.25±1.15	88.22±1.15	79.25±1.3	89±1.6 ^{E}	76.75±1.3^{F}
TG (mmol/L)	0.17±0.01	0.13±0.02	0.14±0.01	0.12±0.01	0.13±0.01	0.12±0.01	0.12±0.01	0.11±0.01
NEFA (mEq/L)	0.56±0.02 ^{EA}	0.35±0.03 ^{F}	0.51±0.03^{EA}	0.3±0.04 ^{F}	0.37±0.02	0.27±0.03	0.23±0.04^{B}	0.24±0.04

The statistical analysis was performed using the MIXED procedure of SAS for repeated measures (SAS User's Guide Statistics, 1999) with the animal as random effect.

The fixed effects of treatment and time were evaluated, as well as that of the interaction time * treatment.

The fixed effect of farms was included only in the initial draft of the statistical model; in fact it did not make a significant contribution so it was not considered in the final model.

The Scheffè test was used for multiple testing.

As to pregnancy rate, a chi-squared test was used.

A value of $P<0.05$ was set as significant level.

RESULTS

Mefepronic acid didn't exert any side effect. All the cows were free from clinical disease or disorder. BCS at calving was 3.5 ±0.3 and decreased to 2.75 ±0.2, on Day 40 *post partum*, in all cows. Liver biopsy was easily performed, without any complication.

Biochemical Parameters

Table **1** indicates the values obtained for each biochemical parameter in treated and control groups from D3 to D10, that is after one, two and three treatments, respectively. The values of the biochemical parameters recorded from D15 to D40, that is after the third treatment, are reported in Table **2**. All parameters ranged in the physiologic limits of the bovine species [18].

The comparison of glucose, total proteins and NEFA, between the treated and control group gave rise to statistically significant differences. Particularly, glucose concentrations were higher in MA than in CTL group at D3 and D5 ($P<0.01$); TP were higher in MA than in CTL group at D10 ($P<0.01$); higher NEFA concentrations were detected in MA than in CTL group at D10 and D15 ($P<0.05$).

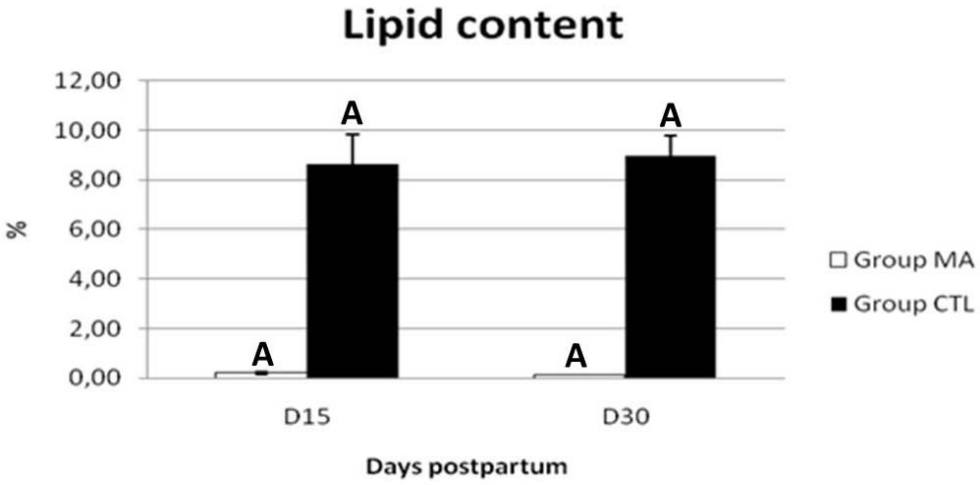

Fig. (1). Percentage of liver cell volume occupied by lipids at D15 (15 days *post partum*) and D30 (30 days *post partum*). Group MA (cows treated with mefepronic acid) *vs* Group CTL (control cows): A, $P<0.001$.

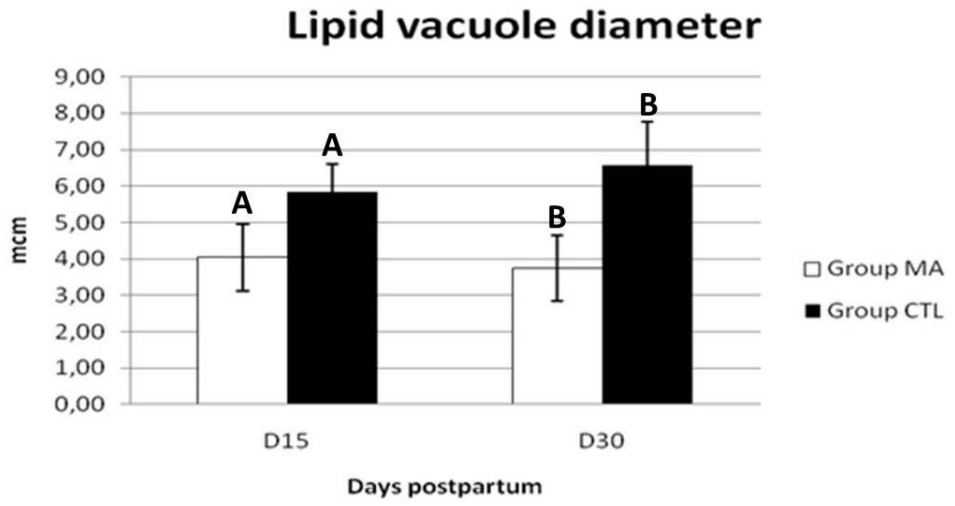

Fig. (2). Diameter (mean ± s.e. from 6 fields, n=20) of hepatic lipid vacuoles at D15 (15 days *post partum*) and D30 (30 days *post partum*). Group MA (cows treated with mefepronic acid) *vs* Group CTL (control cows): A, $P<0.01$; B, $P<0.001$.

Within the same group and in both of them, statistically significant differences were noted for ALT, CHOL, HDL and NEFA, from D10 to D40, as shown in Table **2**.

Histological and Histochemical Evaluation of the Liver

All cows had a moderate fatty liver, according to the reports of Bobe *et al.* [8]. Statistically significant differences were found between the hepatic lipid content of the MA and CTL groups at D15 and D30 (P<0.001) (Fig. **1**).

In Group MA, there was a significant reduction in the diameters of lipid vacuoles at D15 (*P*<0.01) and D30 (*P*<0.001) compared to Group CTL, in which the lipid droplets of the hepatocytes increased in diameter throughout

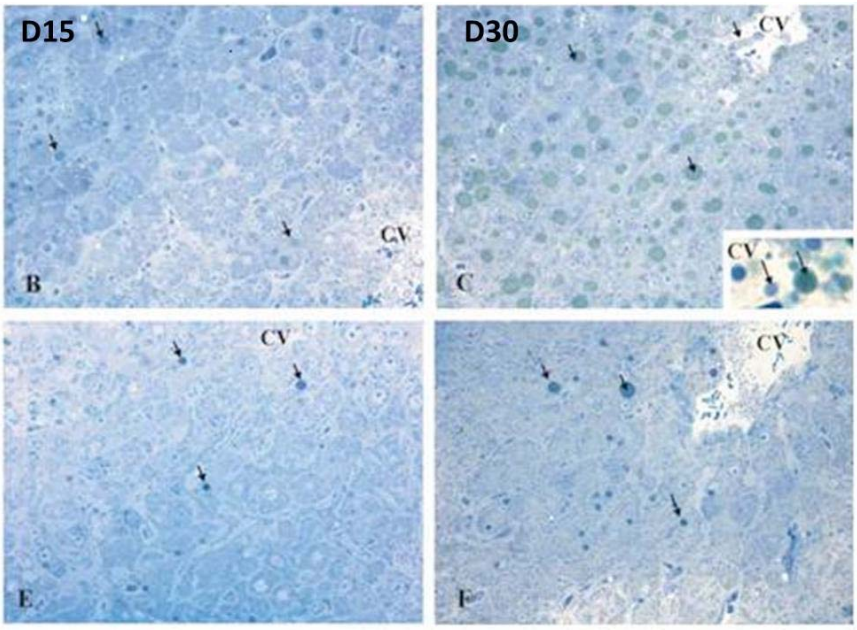

Fig. (3). Semi-fine sections of bovine liver stained with toluidine blue, at D15 (15 days *post partum*) and D30 (30 days *post partum*), to evidence lipid content. B,C: Group CTL (control cows); E,F: Group MA (cows treated with mefepronic acid). CV: centrolobular vein; arrows: lipid vacuoles.

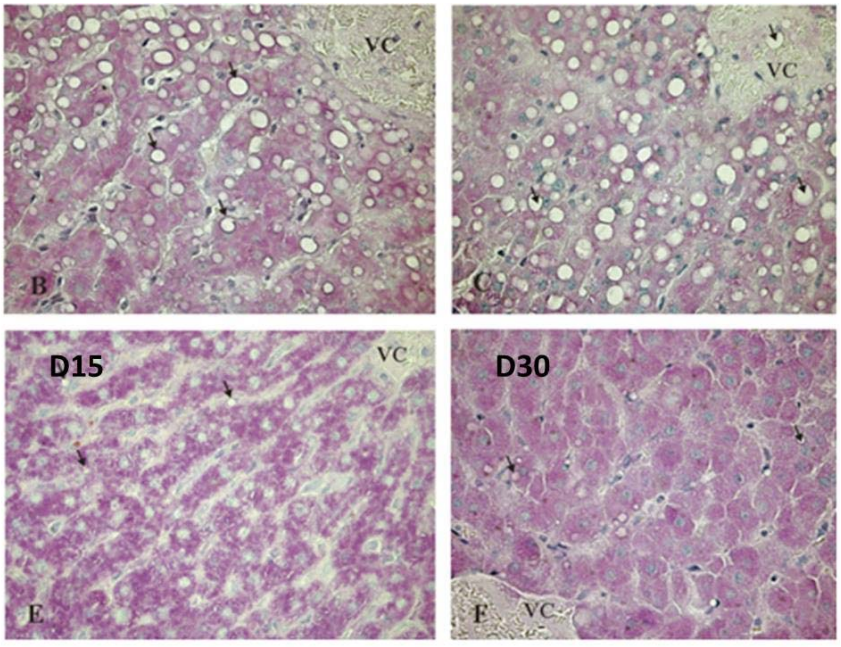

Fig. (4). Paraffin-embedded liver sections stained with Ematossilin-PAS, evidencing glycogen content (Magenta red), at D15 (15 days *post partum*) and D30 (30 days *post partum*). B,C: Group CTL (control cows); E,F: Group MA (cows treated with mefepronic acid). CV: centrolobular vein; arrows: lipid vacuoles.

the experiment, ranging from 5.83±0.25 µm (D15) to 6.58±0.38 µm (D30) (Fig. **2**, Fig. **3**).

As to glycogen, a gradual decrease in the intensity of staining during the experimental period was evidenced in the control sections (Fig. **4**, **B**, **C**). On the other hand, the treated samples showed a more diffuse glycogen content and an augment in the intensity of its staining at D30 compared to those performed at D15 (Fig. **4**, **E**, **F**). Moreover, a difference in glycogen content was reported between the two groups at D30, with Group MA showing the highest quantity.

Immunohistochemical Detection of PPARα

PPARα were detected in the nuclei of hepatocytes. In both groups, the number of immune-positive cells gradually decreased from D15 to D30, even if not significantly. It's noteworthy that the number of cells showing positivity for

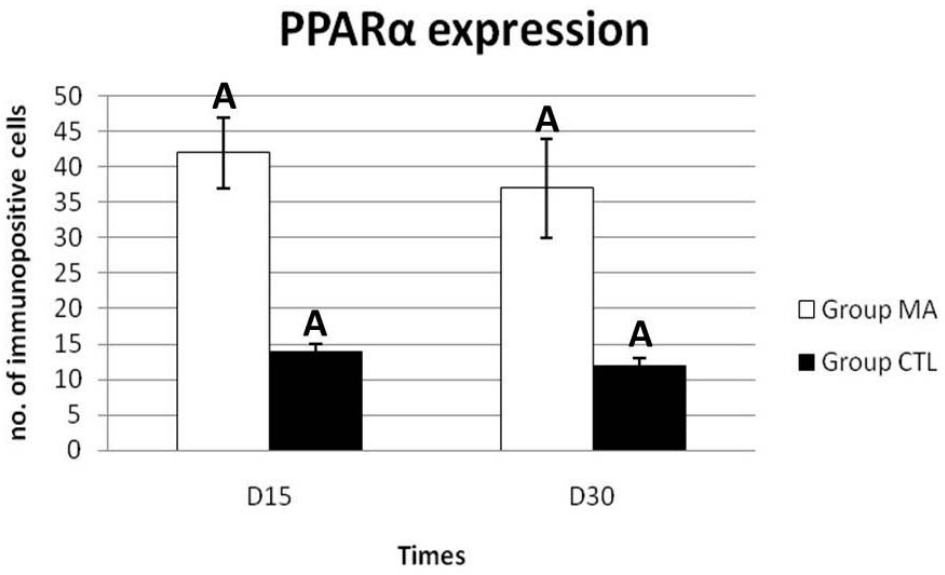

Fig. (5). PPARα immune-positive hepatocytes at D15 (15 days *post partum*) and D30 (30 days *post partum*) in Group MA (cows treated with mefepronic acid) and Group CTL (control cows). Group MA *vs* Group CTL: A, $P<0.01$.

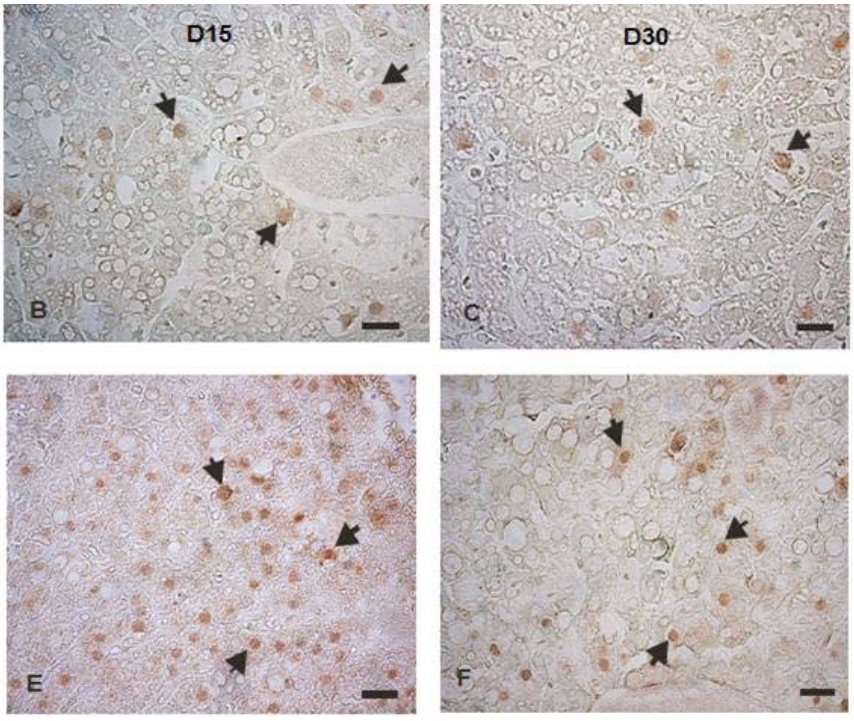

Fig. (6). Immunohistochemical detection of PPARα on bovine liver. Immune-reactivity is localized at the nuclear level (arrows). Bar: 30µm. B,C: Group CTL (control cows); E,F: Group MA (cows treated with mefepronic acid).

Table 3. Progesterone (P4) concentration at T11 and T13 (11 and 13 days after Artificial Insemination) and reproductive parameters in Group MA (treated) and Group CTL (control group). Data are expressed as mean ±S.D., n=30. A,B: P<0.05.

	P4 T11 (ng/mL)	P4 T13 (ng/mL)	Calving-1st estrus (d)	Calving-Conception (d)	% Preg. 1st A.I.	% Preg. 2nd A.I.
Group MA	5.46 ± 0.43	6.47 ± 0.37 A	50 ± 3.7 A	92 ± 2.8 A	57	71
Group CTL	3.70 ± 0.52	4.24 ± 0.37 B	74.36 ± 6.2 B	121.4 ± 3.6 B	50	64

PPARα was significantly higher in group MA than in Group CTL, both at D15 (P<0.01) and D30 (P<0.01) (Fig. **5**, Fig. **6**).

Reproductive Parameters

The effect of treatment on reproductive parameters is shown in Table **3**. Control cows displayed their first oestrus 74.36±6.2 days *post partum*, whereas in the treated cows the first oestrus occurred 50±3.7 days *post partum* (P<0.05). The calving-conception interval was 121.4±3.6 days in Group CTL and 92±2.8 days in Group MA (P<0.05). The pregnancy rate was 57% in Group MA and 50% in Group CTL, at the first A.I., whereas it was 71% in Group MA and 64% in Group CTL, at the second A.I.

Thirteen days after A.I., P_4 concentration was statistically higher (P<0.05) in Group MA than in Group CTL (6.47±0.37 *vs*. 4.24±0.37 ng/mL).

DISCUSSION

A complex network of mutual interaction exists between hepatic dysfunction (such as lipidosis) and impaired reproductive efficacy (ovarian activity and pregnancy rate) in the dairy cow [8]. In this view, the support of the hepatic activities through the improvement of its metabolism could be able, *per se*, to ameliorate the reproductive performances of the dairy cow. In this study mefepronic acid, a PPAR-α agonist endowed with the capability of promoting peroxisomal ß-oxidation and hepatic gluconeogenesis [19], was employed, being administered for only three times, until the fifth day postpartum.

The times of administration were chosen based on the needing of supporting the hepatic function in a narrow window of the transition period (early *post partum*), during which the cow faces tremendous metabolic and hormonal rearrangements. Among these drastic changes, an intense peripheral lipo-mobilization and, consequently, a sharp augment in liver lipid content are frequent to occur in this period [20-22]. In this experimentation the biochemical parameters that showed statistically significant differences between the treated and control groups are glucose, total proteins and NEFA.

As to glucose levels in the treated group, the initial and not significant decrease observed at 3 and 5 days postpartum could be due to the effect of mefepronic acid which may have started stimulating insulin secretion by pancreatic ß-cells [23] and, generally speaking, the overall metabolic functions in the organism. From Day 10 *postpartum* onwards, the increase in glycaemia and the contemporary increase in milk production are likely to have depended upon the intense peripheral lipomobilization and the earlier and more efficient restoration of the hepatic functions, more evident in the treated than in the control group [16].

This is inferable on the basis of the progressive increase in glycaemia observed in group MA (likely to derive from the stimulus exerted by mefepronic acid on the hepatic glucogenesis) [24, 25], and of blood NEFA concentrations, which are statistically higher in the treated than in the untreated group, from Day 10 *postpartum* on. Moreover, these findings match with the histological results, which are characterized by a progressive increase in glycogen content in the hepatocytes, not observed in the Group CTL.

On the other hand, the increase in blood total proteins observed in the treated group 10 days after calving compared to the control one and the persistence of their high blood levels until the end of the study in the same group, further mirror the amelioration of the hepatic performances.

From Day 30 *postpartum* on, the absence of statistically significances between NEFA levels and glucose concentrations in the two groups suggests the restoration of the hepatic physiology and of the systemic metabolism may have been completed.

The increasing levels of cholesterol and HDL display an opposite trend compared to NEFA, confirming the progressive improvement of the hepatic metabolism and of lipogenesis.

The effects induced by MA on lipid metabolism reflect the histological results because a progressive decrease in lipid diffusion was observed in the hepatocytes, a finding mainly evident in Group MA.

In this study, an increase in PPARα receptors was observed in Group MA, compared to Group CTL. This datum, together with the biochemical results discussed above, could let infer a likely role of PPARα agonists in the modulation of their own receptors as well as in β–oxidation [16] and, subsequently, on NEFA blood levels.

Probably, as seen for other receptors [26, 27, 28], besides its direct action on PPARα, MA could have up-regulated its own receptors, thus further increasing the hepatic overall oxidative capability. This effect could have been due to a MA-induced increase in PPARα-gene expression, a decrease in PPARα degradation, or both. This, in turn, may have prevented NEFA accumulation in the liver and may have reduced their free blood concentration [29].

The importance of PPARα and peroxisomal β-oxidation for the catabolism of NEFA in peri-parturient dairy cows, has been outlined in several works [30, 31]. In these studies,

it has been shown that in case of high fatty acid mobilization, NEFA or their metabolites can activate PPARα *per se*, thus inducing those enzymes involved in intracellular metabolism of lipids. In this view, the reduction in lipid content observed in the hepatocytes of the treated group may have been determined by MA.

Moreover the overall improvement of the hepatic conditions observed in the treated group, *i.e*., the reduction in hepatocyte lipid and the increase in glycogen content, may have also depended on the likely capability of MA to lower inflammation and immune reactivity, also in the liver. It's well documented, in fact, that PPARα agonists are able to inhibit the induction of interleukin-6 (IL-6) and cyclooxygenase-2 (COX-2) by interleukin-1 (IL-1), through the inhibition of the translocation of the Nuclear Factor-kB (NF-kB) from the cytosol to the nucleus [14], to induce apoptosis of macrophages and to antagonize NF-kB activation in T cells and B lymphocites, in the hepatic parenchyma [15]. Furthermore, pro-inflammatory cytokines such as TNF-α and IL-6 have been implicated in the determinism of insulin resistance and steatosis, in human medicine [32].

The amelioration of the reproductive parameters obtained with MA is probably due to the general enhancement of the hepatic functions and to the increase, even if not statistically significant, of cholesterol (known progesterone precursor) [33] in the treated group. This is in fact mirrored by the statistically significant increase in progesterone concentrations in the MA group, compared to the control one. Moreover, the treatment may have ameliorated uterine and tubal micro-environment, ovarian function, the quality of oocytes, thus decreasing the incidence of *post partum* diseases [34-36].

This study highlights the benefits of MA treatment on farm efficiency because of its effects on liver metabolism and reproductive performances. Moreover, our clinical study shows that MA modulates the expression of PPARα in the bovine liver.

Concluding, MA should be considered a veterinary aid useful for ameliorating hepatic histological features and functions, resulting in an improvement of general health and reproductive parameters in *post partum* dairy cows.

CONFLICT OF INTEREST

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ABBREVIATIONS

A.I.	=	Artificial Insemination
ALB	=	albumin
ALT	=	alanine aminotransferase
AST	=	aspartate aminotransferase
BCS	=	Body Condition Score
BHBA	=	beta-hydroxybutyric acid
CHOL	=	cholesterol
DBil	=	direct bilirubin
GGT	=	gamma-glutamyltransferase
GLU	=	glucose
HDL	=	high density lipoprotein
IBil	=	indirect bilirubin
MA	=	mefepronic acid
NEFA	=	non-esterified fatty acids
NGS	=	Normal Goat Serum
PPARs	=	peroxisome proliferator-activated receptors
SAP	=	serum alkaline phosphatase
TBil	=	total bilirubin
TG	=	triglycerides
TP	=	total proteins
ALB	=	Albumin
SAP	=	Serum Alkaline Phosphatase
ALT	=	Alanine Aminotransferase
AST	=	Aspartate Aminotransferase
GGT	=	Gamma-Glutamyltransferase
DBil	=	Direct Bilirubin
IBil	=	Indirect Bilirubin
TBil	=	Total Bilirubin
BHBA	=	beta-hydroxybutyric
CHOL	=	Cholesterol
GLU	=	Glucose
HDL	=	High Density Lipoprotein
TP	=	Total Protein
TG	=	Triglycerides
NEFA	=	Non Esterified Fatty Acid
MA	=	group treatment
CTL	=	group control
D3	=	3 days after calving
D5	=	5 days after calving
D10	=	10 days after calving
D15	=	15 days after calving
D30	=	30 days after calving
D40	=	40 days after calving

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