ORIGINAL PAPER

The Effect of Apigeninin Experimentally Induced Acute Pancreatitis. Evaluationof Interleukin-6, Interleukin-18, Resistinin Serum andHigh Mobility Group Box 1 Proteinand CD45 Expressions in Intestinal tissue

Sovatzidis Apostolos[1],Oikonomou Panagoula[1,2], Nikolaidou Eirini[3], Lampropoulou Maria[4], Papachristou Fotini[5], Papalois Apostolos[6], Manousi Maria[1], Tsalikidis Christos[1,2], Pitiakoudis Michael[1,2], Tsaroucha K Alexandra[1,2,5]

[1]Postgraduate Program in Hepatobiliary / Pancreatic Surgery, Faculty of Medicine, Democritus University of Thrace, Alexandoupolis, Greece,68100

[2]2nd Department of Surgery, Faculty of Medicine, Democritus University of Thrace, Alexandroupolis, Greece, 68100

[3]Department of Plastic, Reconstructive and Hand Surgery & Burns ICU, General Hospital of Thessaloniki "G. Papanikolaou", Thessaloniki, Greece, 57010

[4]Laboratory of Histology and Embryology, Faculty of Medicine, Democritus University of Thrace, Alexandroupolis, Greece, 68100

[5]Laboratory of Experimental Surgery and Surgical Research, Faculty of Medicine, Democritus University of Thrace, Alexandroupolis, Greece, 68100

[6]Experimental, Educational and Research Center ELPEN, 19009

ABSTRACT

BackgroundApigenin (AP) is a flavonoid substance, in nature abundance, which is described to have anti-inflammatory and anti-oxidative effects. We investigated the effect ofAPon acute pancreatitis (ACP)bymeasuring the values ofinterleukin-6 (IL-6), interleukin-18 (IL-18) andresistin (R)in serumandCD45as well as high mobility group box 1 protein (HMGB1)inintestinaltissue using a rat experimentalmodel. **Methods** 126male Wistar rats, (age 90-120 days, body weight 250-350 g), were used. The rats were divided in three groups: Sham (S), Control (C) and Apigenin (Ap).The S group comprised 20 rats, which underwent laparotomy and closure of the abdominal wall. TheC group comprised 56 rats, which underwent ligation of the pancreatic duct to induce acute pancreatitis. The apigeningroup comprised 50 rats, which, after induction of ACP, received AP. Each group was divided into 5 subgroups according to the time euthanasia was performed (i.e., 6, 12, 24, 48 and 72 hours after laparotomy).Blood and intestinal tissue were collected.**Results**Comparing the C with theAp group we observed an improvement in IL-6 and IL-18 examined in serum and in HMGB1 as well as in CD45 expression in intestinal mucosa. Following APadministration IL-6 and IL-18 decreased at 12h in the Apgroup. HMGB1 increased significantlyin C group and decreased remarkably in Apgroup over time. CD45 decreased at 24h after APadministration in the Ap group. In contrast to the previous results R remained at low levels in the Apgroup.**Conclusions**APadministration in rats in a bilio-pancreatic duct ligation experimental model ofACPappears to have a protective effect on intestine and serum parametersreducing the severity of ACP. Therefore administration of that substance in humans could improve the damage of the organs affected in the early stages of ACP.

Received September 01st, 2020 -Accepted September 23rd, 2020
Keywords Apigenin; HMGB1; Interleukin-6; Interleukin-18; Pancreatitis;Resistin
AbbreviationsACP Acute Pancreatitis; APApigenin; Ap groupApigenin group; C group Control group; Damage Associated Molecular PatternDAMP; HMGB1High Mobiity Group Box 1; IL-6Intereukin-6; IL-18Intereukin-18;Janus Kinase/Signal Transducer and Activator JAK/STAT;RResistin; S groupSham group; TCM Traditional Chinese Medicine; TLRs Toll-Like Receptors
Correspondence SovatzidisApostolos
Postgraduate Program in Hepatobiliary / Pancreatic Surgery
Faculty of Medicine, Democritus University of Thrace
Alexandoupolis, Greece, 68100
Tel+306936143424
E-mailt.sovatzidis@gmail.com

INTRODUCTION

ACP is characterized by inflammation of the pancreas with involvement of neighboring tissues or distant organs/systems. In mild form, there is recovery without complications. In severe form, there are local and systemic complications such as renal and pulmonary failure as well as bowel related complications [1,2]. Although the exact mechanisms that initiate an episode of ACP are not clearly understood, all cases share the same inflammatory and repair pathways. Unregulated activation of trypsin within pancreatic acinar cells causes local inflammation and leukocyte turnout at the peripancreatic vascular network.

Within a few hours activated monocytes are attracted, further stimulating local and systemic hyperproduction of pro-inflammatory cytokines and interleukins such as IL-6 and IL-18 [3,4]. HMGB1 and CD45 play an important role in mediating inflammation and regulating immune responses, respectively. HMGB1 acts as pro-inflammatory cytokine when released extracellularly under stress conditions, like ACP [5,6]. CD45 is present on pancreatic acinar cells having various roles in activation-induced signaling in leukocytes, related to the inflammatory responses [7,8]. Moreover, according to recent studies, a new effective indicator that can predict the severity of ACP and is used for its assessment is R [9]. Studies in early stages of ACP have difficulties in being performed on humans, as the diagnosis is established when pancreatic injury has already been initiated. As a consequence there is missing information about the facts that occur during the initial stages of ACP. This gap can be filled through research on experimental animal models of ACPin order to enrich our knowledge [10]. The beneficial role of AP, a flavonoid substance, has been described in several studies [11]. It is proven that has anti-inflammatory, anti-oxidative, anti-carcinogenic, anti-mutant, anti-allergic and anti-osteoporotic effects [12]. The aim of this study is to determine whether AP has positive effect on experimentalmodel of ACP. In order to accomplish it, we determined IL-6, IL-18 and R levels in serum and HMGB1 and CD45 expression in intestinal tissue of animal models with ACP after AP administration at different time points.

MATERIALS AND METHODS

Animals and Study Design

126albine male wistar rats, 90-120 days old, with a median weight of 280g (IQR=220-350g), were used. The animals were maintained in groups of four, in polycarbonate cages, under controlled conditions of temperature (22°C – 25°C), humidity (55%-58%) and light (12/12 hours light/dark) with free access to rat food and tap water ad libitum.

All animals were supplied by Pasteur Institute, Athens, Greece. The experiments took place at the approved Experimental Research Center of ELPEN Pharmaceuticals, Athens, Greece, and the immunohistochemistry and ELISA assays took place at the laboratories of Medical School of Democritus University of Thrace. All experimental procedures conformed to the principles laid by the National Research Council Guide for the Care and Use of Laboratory Animals and the Directive 86/609 of the European Union (protocol number K/2284).

The experimental animals were randomly assigned into 3 groups: 1) S group (S; n=**20**), in which animals underwent midline laparotomy without induction of ACP or AP administration;2) C group (C; n=**56**), in which animals underwent midline laparotomy with induction of ACPvia ligation of the common bilio-pancreatic duct; and 3) Ap group (Ap; n=**50**), in which animals underwent midline laparotomy with induction of ACP, followed by postoperative per osadministration of AP. The animals of each group were further subdivided into five subgroups according to the euthanasia time, i.e., 6h (C6, Ap6),12h (C12, Ap12),24 h (C24, Ap24),48h (C48, Ap48) and 72h (C72, Ap72). In the Apgroup, the substance was administered as follows: i) Ap_6 (n=10): 1 dose of 4mL, ii) Ap_{12} (n=10): 1 dose of 4mL,iii) Ap_{24} (n=10): 1 dose of 4mL, iv) Ap_{48} (n=10): 2 doses of 4mL with a 24 h interval, v) Ap_{72} (n=10): 3 doses of 4mL with 24 h intervals, followed by euthanasia. The overall study design was based on a previous publication by members of our research group [13].

Protocol of Experimentally Induced ACPin Rats

Induction in anaesthesia was performed by placing each experimental animal in a special glass box connected to isoflurane supply for about 2 to 3 min, followed by subcutaneously injected 0.25mL of Butorphenol 10 mg/mL (Dolorex;Intervet/Schering/Plough Animal Health, Boxmeer, Holland). Endotracheal intubation was performed under direct laryngoscopy by trained staff using a 16-G venous catheter connected to an Ugo-Basile ventilator for rodents (Harvard Apparatus, Holliston, MA), which was adjusted at tidal volume of 3 mLand respiratory rate of 70 breaths/min. Chest expansion and lung auscultation was indicating a proper intubation.

With the experimental animal placed in a supine position, a midline laparotomy of about 3cm was performed under sterile conditions. After the entry in the abdominal cavity the bilio-pancreatic duct was identified and ligated close to the duodenum with a non-absorbable 4/0 silk suture for the induction of ACP [14]. 1 mL of NaCl 0.9% was instilled in the abdominal cavity before closing the lineaalba with vicryl 3/0 suture and the skin with nylon 3/0 suture. Postoperatively the animal was fed ad libitum while analgesia (2mL ofbutorphenol, Dolorex) was administered subcutaneously every 4 hours, and then, according to the animal's needs based on their clinical picture. In S group, operation was followed by identification and manual mobilization of the pancreas without ligation of the bilio-pancreatic duct or further action before the abdominal closure. In Apgroup, after the surgical ligation of the duct, once the animal was becoming conscious, 4 mL of prepared AP solution was administered orally.

Nine rats died before scheduled euthanasia, two during the surgical experiment due to intubation complications from the Cgroup and seven post-surgery, one from the Apgroup and six from the Cgroup. These animals were not included in the statistical analysis.

AP Solution Preparation

AP was suppliedby Sigma-Aldrich, Taufkirchen, Germany in vials with >99% purity by thin layer chromatography, as yellow powder with tan cast. AP was dissolved as follows: 660 mg AP was dissolved in 500mL corn oil, 2mL dimethyl sulfoxide and 10mL Tween 80. The final concentration was 5 mg AP in 4mL solution.

Immunohistochemistry

The immunochemistry procedure was performed in paraffin-embedded tissue section by using the biotin / streptavidin technique. The kits that were used were Super Sensitive Onestep Polymer HRP detection system (QD 630XAKE, Biogenex) for HMGB1 and CD45. The antibody used for the detection of HMGB1 was the HMGB1 polyclonal antibody in dilution 1:250 and incubation time 60min (rabbit polyclonal, PA185-16926, Thermo Scientific) and forCD45 was the monoclonal antibody in dilution 1:250 and incubation time 60min (mouse monoclonal, sc53047, Santa Cruz). A scoring system for all antibodies was assigned according to the proportion of cells with cytoplasmic staining as follows:i) 0: negative (≤10% of stained cells); ii) 1:low (>10% and ≤30% of cells stained); iii) 2: moderate (>30% and ≤70% of cells stained) and iv)3: high (>70% cells stained).

Determination of Serum IL-6,IL-18and Resistin

IL-6, IL-18and resistinprotein levels were determinated in serum samples by ELISA according to the manufacturer's instructions (*IL-6*: rat IL-6, ER 2116, Thermo Scientific, USA;*IL-18*: rat IL-18, orb 50132, biorbyt, UK;*resistin:* E02R0351, amsbio, UK). Protein levels were interpolated from the corresponding standard reference calibration curves. BCA protein assay (Thermo Scientific, USA) was used for total protein determination.

Statistical Analysis

The continuous variables are demonstrated as median with range (min-max). The non-parametric tests Mann-Whitney and Kruscal-Wallis were used to compare the medians of continuous variables in different categories of qualitative variables (group and time). In the case of a statistically significant result of Kruscal-Wallis test, Dunn's post hoc tests were carried out on each pair of groups. The Bonferroni adjustment was used. As post-hoc tests are more conservative than the global test there was a case where the results of Kruscal-Wallis were statistically significant but pairwise comparisons were not. Moreover, if the number of observations in each group or time point was less than four, a sensitivity analysis was conducted keeping only the groups and time points with more than four observations. As the study variables were classified into multiple categories, the non-parametric tests were used due to the small samples. Relationships with a P-value (p) ≤ 0.05 were considered as statistically significant. All reported P-values are two-sided. The data was analyzed in using the Statistical Package for the Social Sciences 25.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

AP ReducesIL-6in Rats with Experimental ACP at 12h

IL-6 concentrations were lower in S group compared to C andAp groups, at every time point**(Figure 1)**. More specifically, in S group, the initial value was the highest (mean: 61.10; range: 30.80 – 64.70) and diminuated gradually for every time point that followed**(Figure 1)**.In C group IL-6 reached its highest value at 12h (mean: 129.30;range: 63.70 – 263.50) due to the inflammation caused by ligation of the bilio-pancreatic duct. At this time point of 12h, values in C and Ap groups almost coincided and presented their highest levels (mean: 162.50; range: 110.90 – 311.60). It is shown that after 12h, in the Apgroup the level of IL6 is reduced, leading to a steady decline, whereas in the C group, fluctuations of the level of IL6 are seen.Probably, the beginning of the anti-inflammatory effect of the substance starts at 12h, result which also reaches statistical significance from the pairwise comparisons between S and Ap groupsat 12h (p=0.007) and at 24h(p=0.005) **(Table 1)**. From comparisons between the 5 time points for each group, in the Ap group, every time point combination presented statistical significant results (p<0.001), underlying the effect of APadministration **(Table 2)**.

AP Reduces IL-18 in Rats with Experimental ACP at 6 h

IL-18 remained at low levels in the S group, with a peak at 24 h time point **(Figure 2)**. In the C group, it reached the highest value at 6 h time point, diminished until 12 h and then augmented until 48 h, where it started to fall again. In the Ap group, the onset value was the highest of all 3

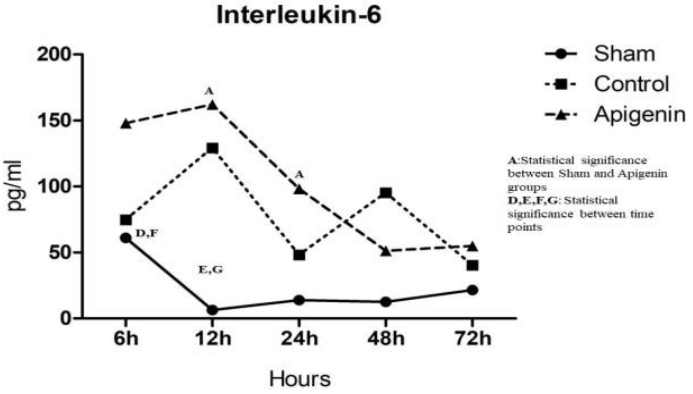

Figure 1. Changes inIL-6 in three groups over the 72h period.(A). indicates statistical significance between Sham and Apigenin groups at 12 (p=0,007) and 24 hours (p=0,005). (D). indicates statisticalsignificance between 6-72 hours (p=0,001)at the Apigenin group. (E). indicates statisticalsignificance between 12-72 hours (p=0,001) at the Apigenin group. (F). indicates statisticalsignificance between 6-48 hours (p=0,001) at the Apigenin group. (G). indicates statisticalsignificance between 12-48 hours (p=0,001) at the Apigenin group. Significance was set at p<0,05. Data are presented as median values.

Table 1. Between group differences over the 5 time points.

Variable	Time points	SHAM	CONTROL	APIGENIN	*p-value for between groups*
Interleukine 18	**6H**	0.09 (0.01-0.45)	1.56 (0.01-3.33)	2.03 (0.22-3.68)	**0.028**[A]
	12H		0.40 (0-3.57)	2.15 (1.18 -2.94)	**0.038**
	24H		0.85 (0.23-1.80)	0.57 (0-2.09)	0.662
	48H		0.95 (0-5.03)	0.97 (0 -2.26)	0.798
	72H	0.03 (0-0.52)	0.18 (0-2.24)	0.54 (0-7.33)	0.143
Resistin	**6H**	0.15 (0.10-0.26)	0.19 (0.05-0.31)	0.12 (0.01-0.23)	0.422
	12H		0.31 (0.02-0.75)	0.07 (0.04-0.14)	**0.01**
	24H		0.22 (0.06-1.06)	0.14 (0.08-0.25)	0.234
	48H		0.44 (0.14-0.72)	0.19 (0.11-0.36)	**0.028**
	72H	0.21 (0.04-0.26)	0.25 (0.08-0.51)	0.17 (0.13-0.39)	0.582
CD45	**6H**	1 (1-1)	2 (1-2)	2 (1-2)	**0.049**[A]
	12H	1 (1-1)	1.50 (1-2)	2 (1-2)	0.127
	24H	1 (1-1)	2 (1-3)	1 (1-2)	**0.043**[B]
	48H	1 (1-1)	2 (2-3)	2 (1-3)	**0.002**[B,C]
	72H	1 (1-1)	3 (2-3)	1 (0-1)	**<0.001**[B,C]
HMGB1	**6H**	0.50 (0-1)	2 (2-3)	3 (2-3)	**0.002**[A,B]
	12H	0.50 (0-1)	2 (2-3)	2 (1-2)	**0.002**[B]
	24H	1 (0-1)	3 (2-3)	1 (1-2)	**0.001**[A,B]
	48H	1 (1-1)	3 (2-3)	1 (0-1)	**<0.001**[B,C]
	72H	1 (1-1)	3 (3-3)	0.50 (0-1)	**<0.001**[C]
Interleukine 6	**6H**	61.10 (30.80-64.70)	74.80 (37.20-332.00)	147.90 (83.10-264.90)	0.053
	12H	6.37 (3.49-33.70)	129.30 (63.70-263.50)	162.05 (110.90-311.60)	**0.007**[A]
	24H	13.94 (1.41-21.50)	48.20 (23.40-223.20)	98.25 (44.70-765.30)	**0.005**[A]
	48H		95.20 (27.80-127.30)	51.25 (33.70-76.40)	**0.015**
	72H		40.45 (20.06-171.60)	54.90 (14.60-83.10)	0.878

[A]Sham*vs.*Apigenin;[B]Sham*vs.* Control; [C]Control*vs.*Apigenin

Table 2: Within group differences over thetime points.

Variable	Group	6H	12H	24H	48H	72H	*p-value for between time points*
Interleukine 18	**SHAM**	0.09 (0.01-0.45)				0.03 (0-0.52)	0.686
	CONTROL	1.56 (0.01-3.33)	0.40 (0-3.57)	0.85 (0.23-1.80)	0.95 (0-5.03)	0.18 (0-2.24)	0.129
	APIGENIN	2.03 (0.22-3.68)	2.15 (1.18 -2.94)	0.57 (0-2.09)	0.97 (0 -2.26)	0.54 (0-7.33)	**0.032**
Resistin	**SHAM**	0.15 (0.10-0.26)				0.21 (0.04-0.26)	0.886
	CONTROL	0.19 (0.05-0.31)	0.31 (0.02-0.75)	0.22 (0.06-1.06)	0.44 (0.14-0.72)	0.25 (0.08-0.51)	0.196
	APIGENIN	0.12 (0.01-0.23)	0.07 (0.04-0.14)	0.14 (0.08-0.25)	0.19 (0.11-0.36)	0.17 (0.13-0.39)	**0.002**[E,G]
CD45	**SHAM**	1 (1-1)	1 (1-1)	1 (1-1)	1 (1-1)	1 (1-1)	1
	CONTROL	2 (1-2)	1.50 (1-2)	2 (1-3)	2 (2-3)	3 (2-3)	**0.001**[D,E]
	APIGENIN	2 (1-2)	2 (1-2)	1 (1-2)	2 (1-3)	1 (0-1)	**0.011**[D,E]
HMGB1	**SHAM**	0.50 (0-1)	0.50 (0-1)	1 (0-1)	1 (1-1)	1 (1-1)	0.281
	CONTROL	2 (2-3)	2 (2-3)	3 (2-3)	3 (2-3)	3 (3-3)	**0.001**[D,F]
	APIGENIN	3 (2-3)	2 (1-2)	1 (1-2)	1 (0-1)	0.50 (0-1)	**<0.001**[G]
Interleukine 6	**SHAM**	61.10 (30.80-64.70)	6.37 (3.49-33.70)	13.94 (1.41-21.50)			**0.036**
	CONTROL	74.80 (37.20-332.00)	129.30 (63.70-263.50)	48.20 (23.40-223.20)	95.20 (27.80-127.30)	40.45 (20.06-171.60)	0.077
	APIGENIN	147.90 (83.10-264.90)	162.05 (110.90-311.60)	98.25 (44.70-765.30)	51.25 (33.70-76.40)	54.90 (14.60-83.10)	**<0.001**[D,E,F,G]

[D]6Hvs 72H; [E]12Hvs 72H; [F]6Hvs 48H; [G]12Hvs 48H

groups (mean:2.03; range: 0.22-3.68) **(Table 2)**. The effect of APstarted at 12h, followed by a huge drop in value of IL-18 until 24 h, when it rose again. Another drop appeared at 48h with the lowest value at 72h. The only statistically significant result was at the onset point (p=0.028), in which Ap group showed the highest and the S group, one of the lowest values **(Table 1)**.

AP Reduces HMGB1 Expression

HMGB1 expressions among the 3 groups reached statistically significant differences almost at all-time points **(Table 1)**. HMGB1 presented low expressions in the S group, whereas in the C group, it increasedafter 12 h and reached its highest value at 24h **(Table 2,Figure 3)**. The beneficial role of APstarted at 48h (p=0.02), while

HMGB1 gained its lowest value in theAp group at 72 h time point (p=0.001) **(Table 1)**, with statistical significance in pairwise comparison of 6h *vs.* 72 h (p<0.001) **(Table 2)**.

AP PresentsAnti-Inflammatory Effect, as CD45Expression is Decreased

In the S group, CD45 remained low and stable at every time point **(Figure 2)**. In the C group, it increased reaching its highest value at 72 h. On the contrary, in the Ap group, the expressions of CD45 coincidedwith those of the S group after 24h. Statistical significance for pairwise comparisons between the S and C groupswas found for every time point after 24h (24h: p=0.043; 48h: p=0.002; 72h: p<0.001) and between C and Ap at 48h (p=0.002) and 72 h (p<0.001) **(Table 1)**. From the comparisons between the 5 time points for each group, both C and Ap groups presented statistical significant results for 6h *vs.* 72h (p=0.001) and 12h *vs.* 72h (p=0.011), underlying for the C group the upward trend of values and for the Ap group the downward trend of values**(Table 2, Figure 4)**.

AP ImprovesInflammatory Effect, as Levels of RRemain at Low Points

In both S and C groups, Rranged from low to high values without any specific pattern **(Figure 5)**. On the contrary, the Ap groups presented the lowest values compared to C

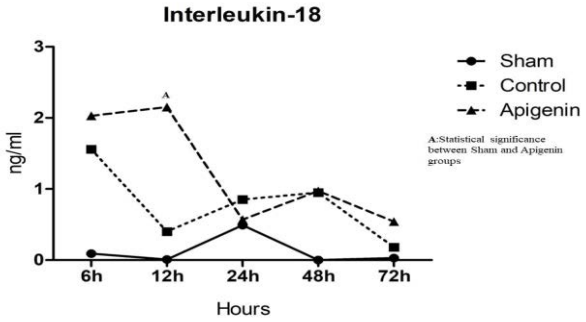

Figure 2. Changes in IL-18 in three groups over the 72h period.(A): indicates statistical significance between Sham and Apigenin groups at 12 hours (p=0.028). Significance was set at p<0,05. Data are presented as median values.

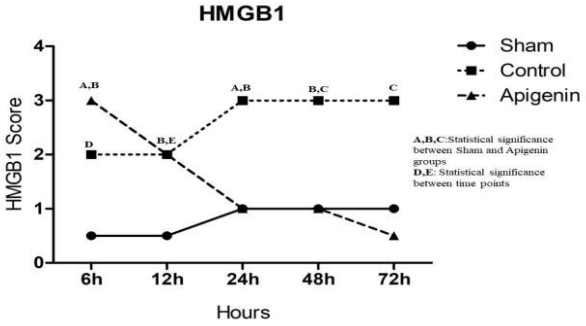

Figure 3. Changes in HMGB1 expression in three groups over the 72h period.(A): indicates statistical significance between Sham and Apigenin groups at 6 (p=0,002) and 24 hours (p=0,001). (B): indicates statistical significance between Sham and Control groups at 6 (p=0,002),12(p=0,002), 24 (p=0,001) and 48 hours (p=0,001),(C): indicates statistical significance between Control and Apigenin groups at 48 (p=0,001) and 72 hours (p<0,001). (D): indicates statisticalsignificance between 6-72 hours at the Control(p=0,001) and Apigenin group(p<0,001). (F): indicates statisticalsignificance between 6-48 hoursat the Control group (p=0,001). Significance was set at p<0,05. Data are presented as median values.

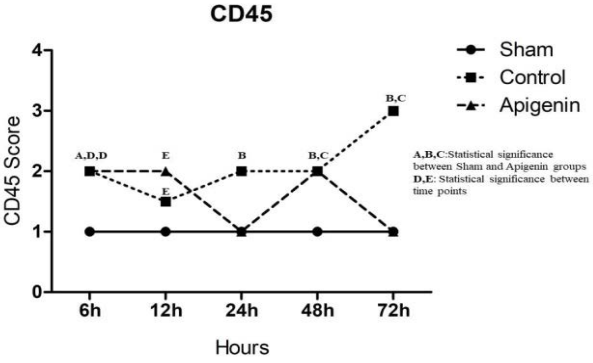

Figure 4. Changes in CD45 expression inthree groups over the 72h period.(A). indicates statistical significance between Sham and Apigenin groups at 6 (p=0,049). (B). indicates statistical significance between Sham and Control groups at 24 (p=0,043), 48 (p=0,002) and 72 hours (p<0,001),(C). indicates statistical significance between Control and Apigenin groups at 48 (p=0,002) and 72 hours (p<0,001). (D). indicates statisticalsignificance between 6-72 hours at the Control (p=0,001) and Apigenin group (p=0,011). (E). indicates statisticalsignificance between 12-72 hours at the Control (p=0,001) and Apigenin group (p=0,011). Significance was set at p<0,05. Data are presented as median values.

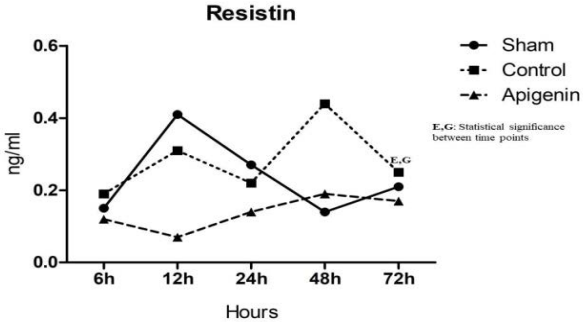

Figure 5. Changes inresistin in three groups over the 72h period.(E). indicates statisticalsignificance between 12-72 hours at the Apigenin group (p=0,002). (G). indicates statisticalsignificance between 12-48 hours at the Apigenin group (p=0,002). Significance was set at p<0,05. Data are presented as median values.

groups, with statistical significance at 12 h, 48h and 72h (p=0.002).

DISCUSSION

ACP is an inflammatory process of the pancreas with adverse outcome. It is characterized by damage of peri-pancreatic tissues and/or distant organs, and it seems to rather comprise a variety of diseases than a sole one [15]. Pathophysiological mechanisms involved in the cause of ACP, especially these of the early stages of the disease, are under study. All mechanisms lead to release of cytokines and free radicals of oxygen, increasing the inflammatory process, the apoptosis and the oxidative stress [16].

Due to the continuous research interest on pancreatitis, severalexperimental models of pancreatitis have been developed. In our study, we used the experimental model of ligation of the bilio-pancreatic duct [14] which mimics acute obstruction similar to gallstone pancreatitis in humans.

Our study refers to the effect of a flavonoid, AP, on known inflammation indices such as IL-6 [3], IL-18 [4], HMGB1 [17], CD45 [18] and R [9] in rat serum and intestinal tissue. Flavonoids are phenolic phytochemicals, naturally existing in plants and abundant in particular plant species, vegetables and fruits. They are considered important constituents of the human diet, although their daily intake varies with dietary habits. The beneficial role of flavonoids is well known in the traditional chinese medicine (TCM). TCM is one of the most popular complementary and alternative medicine modalities worldwide for the treatment of ACP, and one of its most powerful medicines is scutellarin. In the last decade, further studies enhance scutellarin's beneficial effect on inflammation, as a family member of flavonoids [19,20].Several properties have been ascribed to flavonoids including AP. It seems that AP down-regulated Tumor Necrosis Factor–α in an experimental model of ACP, so it is expected, to also down-regulate other inflammatory cytokines [21]. AP is characterized by anti-cancerous, anti-oxidative, anti-inflammatory and other actions [12].

In our study,AP, showed its anti-inflammatory effect, mainly at 12 h reducing IL-6 serum levels. Concerning IL-6, it acts via gp130 protein, leading the activation of Janus kinase/signal transducer and activator (JAK/STAT) signaling pathway [22,23]. In vivo studies proved that patients suffering from pancreatitis developed high serum values of IL-6 compared to healthy individuals (52).A recent study of Mrazeket al.which is in accordance with our findings showed that AP reduces IL-6 levels [11].

IL-18, is a member of IL-1 family cytokines. There are many studies that prove the presence of IL-18 in the blood of patients suffering from ACP. Moreover its values increase during mild and severe forms of the disease compared to healthy people [4]. In experimental models of ACP, high levels of IL-18 have also been described [24].In our study, APshows its anti-inflammatory action between 12h and 24h, as we observe an important reduction of IL-18 value in Ap group. In general, IL-18 seems to be present at the early stages of ACP acting as immunomodulator of the inflammatory response especially in severe forms of the disease, however, without knowing the exact mechanisms of pathogenesis [24].

HMGB1 is a nuclear protein which regulates transcriptional process and genetic expression, while it plays an important rolein replication and repair of DNA. Loss of HMGB1 leads to structural and functional anomalies of the nucleus. Under stress conditions it is moved from nucleus to cytoplasm, where it mediates autophagy. Beyond its intracellular action, it can acts as extracellular protein as well, and more specifically as damage associated molecular pattern(DAMP) molecule. These molecules act as cytokines which through HMGB1 receptors or toll-like receptors (TLRs), activate specific signaling pathways mediating the inflammatory and immune response. According to experimental studies, oxidative stress is responsible for the translocation, release and action of HMGB1 [25,26].

Studies try to explain the relationship between HMGB1 and ACP. Most of them showed linear correlation of HMGB1 and other inflammatory parameters [25,27], while inone study, a reduction of HMGB1 levels was described through the action of siRNA that inhibited NF-kB, decreasing the inflammation in severe ACP [28]. In general, HMGB1 expression in patients with ACP, seems to increase. Two are the possible mechanisms responsible for this. The first one is "HMGB1 circulation" [29], which is a phenomenon under study. According to this, reduction of HMGB1 through administration of anti-HMGB1 antibody causes reduction of the severity of ACP and vice versa. The second mechanismis based to the fact that in ACP, HMGB1 is released from the injured pancreas as well as from

lungs and intestine that are also affected [30]. The results of our study are in total agreement with the majority of the studies, as in the C group we observed statistically significant increase of HMGB1 expression with time while in the Ap group we detected the positive effect of AP after 48 hours of the experiment. Moreover, at every time of the experiment there was statistically significant difference between two of the groups, undoubtedly pointing at the beneficial role of the substance's administration on the intestinal mucosa [31].

CD45 is another antigen related with inflammation. As it was expected, in S group, CD45 remained low and stable for every time point. On the other hand, in both groups with ACP it increased, coming in line with the existing bibliography [32,33]. The AP started its action at 24hours, by down-regulating the expression ofCD45. To the rest of our knowledge, this is the first study that has noticed a great and undoubtableimpact of AP on CD45 of the intestine mucosa.

Concerning R, the lower levels of its concentration may correlate with the early action of AP. Generally, R is involved in the early stages of inflammation. Daniel et al.observed that R serum concentration in patients with ACP was significantly increased on the first, third and fifth day of hospitalization compared to the control group [34]while Xueet al. indicated in their study thatRmay be associated with the occurrence and development of AP and as a resultthe protein may be a valuable indicator for assessing the severity of ACP [35]. In our studythe beneficial role of APon Rwas also obvious.

As it is well known,ACP is also associated with acute lung and kidney injury.Recent studies have proven that the administration of anti-inflammatory substances, such aseugenoland AP, can protect kidneys and lung injury in an experimental model of ACP in rats [36,37]. In our study, we observedthe beneficial role of AP not only in serum parameters, but also in the intestinal mucosa. It seems that the therapeutic target of ACP is not just the pancreas but also other organs – targets of the disease, such as the intestine, the kidneys, the lungs.The prevention of multiple organ disfunction can be useful in diminishing the morbidity and mortality of the disease.

In the present study 4 mL of AP solution were administered every 24 hours containing 5mg of AP,as described previously. This dose proved to be capable to satisfy our hypothesis that AP is a potent anti-inflammatory agent [13], after ACP. The selection of the dosage was based on previous studies investigating daily dietary exposure of AP to humans [38]. Dosages of 100-200mg/kg or more induce cytotoxicity and produce phenoxyl radicals and reactive oxygen species [39,40].

CONCLUSION

In conclusion, AP administration in a rat experimental model appears to be beneficial on the examined parameters as it reduces theinflammation, caused in that experimental model ofACP. APadministration seems to be a promising therapeutic non-toxic agent which should be further tested in clinical use.

ACKNOWLEDGMENTS

This study was funded with a scholarship by the Experimental, Educational and Research Center of ELPEN Pharmaceuticals (EERCE), Athens, Greece, which also provided the research facilities for this project.

DECLARATION OF CONFLICTING INTEREST

The authors declare that there are no conflicts of interest.

References

1. Frossard JL, Steer ML, Pastor CM. Acute pancreatitis. Lancet 2008;371:143-152. [PMID: 18191686]

2. Bansal A, Gupta P,Singh H, Samanta J, Mandavdhare H, Sharma V, et al. Gastrointestinal complications in acute and chronic pancreatitis. JGH Open 2019; 3:450–455. [PMID: 31832543]

3. Saluja A, Dudeja V,Dawra R,Shah RP. Early Intra-acinar Events in Pathogenesis of Pancreatitis.Gastroenterology 2019; 156:1–15. [PMID: 30776339]

4. Li Z, Yu X, Werner J, Bazhin AV, D'HaeseJG. The role of interleukine-18 in pancreatitis and pancreatic cancer. Cytokine Growth Factor Re 2019;50:1-12. [PMID: 31753718]

5. Shen X, Li WQ. High-mobility group box 1 protein and its role in severe acute pancreatitis World J Gastroenterol 2015; 21:1424-1435. [PMID: 25663762]

6. Weng H, Deng Y, Xie Y, Liu H, Gong F. Expression and significance of HMGB1, TLR4, and NF-κB p65 in human epidermal tumors. BMC Cancer 2013; 13: 311. [PMID: 23803172]

7. Rheinlander A, Schraven B, Bommhardt U. CD45 in human physiology and clinical medicine. Immunol Lett 2018;196:22-32. [PMID: 29366662]

8. Zhou C, Saxon A, Zhang K. Human activation-induced cytidinedeaminase is induced by IL-4 and negatively regulated by CD45: implication of CD45, as a Janus kinase phosphatase in antibody diversification. J Immunol 2003;170:1887-1893. [PMID: 12574355]

9. Yu P, Wang S, Qiu Z,Bai B, Zhao Z, Hao Y, et al. Efficacy of resistin and leptin in predicting persistent organ failure in patients with acute pancreatitis. Pancreatology 2016;16:952-957. [PMID: 27654573]

10. Zhan X, Wang F, Bi Y, Ji B. Animal models of gastrointestinal and liver diseases. Animal models of acute and chronic pancreatitis. J PhysiolGastrointest Liver Physiol 2016;311:G343-55. [PMID: 27418683]

11. Mrazek AA, Bhatia V, Falzon M, Spratt HH, Chao C, Hellmich MR. Apigenin Decreases Acinar Cell Damage in Pancreatitis. Pancreas 2019;48:711-718. [PMID: 31091220]

12. Salehi B, Venditti A, Sharifi-Rad M,Kręgiel D, Sharifi-Rad J, Durazzo A, et al. The Therapeutic Potential of Apigenin. Int J MolSci 2019;20:1305. [PMID: 30875872]

13. Lampropoulos P, Lambropoulou M, Papalois A, BasiosN, ManousiM, Simopoulos C, et al. The role of apigenin in an experimental model of acute pancreatitis. J Surg Res 2013; 183:129-137. [PMID: 23273883]

14. Saloman JL, Albers KM, Cruz-Monserrate Z, Davis BM, Edderkaoui M, Eibl G, et al. Animal models: Challenges and opportunities to determine optimal experimental models of pancreatitis and pancreatic cancer. Pancreas 2019;48:759-779. [PMID: 31206467]

15. Nusso DD, Miconi A, Pierantognetti P. Management of Suspected Acute Inflammatory Pancreatopathies in a 'Real-World'Setting: A Single-Center Observational Study. JOP 2014; 15:175-181. [PMID: 24618444]

16. Lee PJ, Papachristou GI. New Insights into Acute Pancreatitis. Nat Rev GastroenterolHepatol 2019;16:479-496. [PMID: 31138897]

17. Yang R, Tenhunen J, Tonnesen TI. HMGB1 and Histones Play a Significant Role in Inducing Systemic Inflammation and Multiple Organ Dysfunctions in Severe Acute Pancreatitis. Int J Inflam 2017;2017:1817564. [PMID: 28316860]

18. Dios ID, Ramudo L, Garcia-Montero AC, Manso MA. Redox-sensitive modulation of CD45 expression in pancreatic acinar cells during acute pancreatitis. J Pathol 2006;210:234-239. [PMID: 16886168]

19. Li J, Zhang S, Zhou R, Zhang J, Li ZF. Persectives of traditional Chinese medicine in pancreas protection for acute pancreatitis. World J Gastroenterol 2017;23:3615-3623. [PMID: 28611514]

20. Zheng J, Wu J, Chen J, Liu J, Lu Y, Huang C, Hu G, Wang X, Zeng Y. Therapeutic effects of quercetin on early inflammation in hypertriglyceridemia-related acute pancreatitis and its mechanism. Pancreatology. 2016 Mar-Apr;16(2):200-10. [PMID: 26873426]

21. Charalabopoulos A, Davakis S, Lambropoulou M, PapaloisA, Simopoulos C, Tsaroucha A. Apigenin exerts anti-inflammatory effects in an experimental model of acute pancreatitis by down-regulating TNF-α. In vivo 2019; 33:1133-1141. [PMID: 31280202]

22. Lesina M, Wörmann SM, Neuhöfer P, Song L, Algül H. Interleukin-6 in inflammatory and malignant diseases of the pancreas. Semin Immunol 2014;26:80-87. [PMID: 24572992]

23. Manohar M, Verma AK, Venkateshaiah SU, Sanders NL, Mishra A. Pathogenic mechanisms of pancreatitis. World J Gastrointest Pharmacol Ther 2017;8:10-25. [PMID: 28217371]

24. Pini M, Senello JA, Cabay RJ, Fantuzzi G. Effect of diet-induced obesity on acute pancreatitis induced by administration of interleukin-12 plus interleukin-18 in mice. Obesity (Silver Spring) 2010;18:476-81. [PMID: 19696761]

25. Li N, Wang BM, Cai S, Liu PL. The Role of Serum High Mobility Group Box 1and Interleukin-6 -Levels in Acute Pancreatitis: A Meta-Analysis. J Cell Biochem 2018;119:616-624. [PMID: 28618057]

26. Kim H, Kim HJ, Kang JH, Kang BT, Yang MP. Evaluation of serum C-reactive protein and high mobility group box 1concentrations in 22 dogs with acute pancreatitis: a pilot study. Vet Q 2019;39:122-130. [PMID: 31401946]

27. Arriaga-Pizano L, Bosco-Garate I, Martnez-Ordaz JL, Wong-Baeza I, Gutiérrez-Mendoza M, Sánchez-Fernandez P, et al. High Serum Levels Of High Mobility Group Box 1 (HMGB1) and Low Levels of Heat Shock Protein 70 (Hsp70) are associated with Poor Prognosis in Patients with Acute Pancreatitis. Arch Med Res 2018;49:504-511. [PMID: 30947809]

28. Luan ZG, Zhang XJ, Yin XH, Ma XC, Zhang H, Zhang C, et al. Downregulation of HMGB1 protects against the development of acute lung injury after severe acute pancreatitis. Immunobiology 2013; 218:1261-1270. [PMID: 23706497]

29. Yasuda T, Ueda T, Shinzeki M, Sawa H, Nakajima T, Takeyama Y, et al. Increase of high-mobility group box chromosomal protein 1 in blood and injured organs in experimental severe acute pancreatitis. Pancreas 2007; 34:487-488. [PMID: 17446855]

30. Mullie P, Clarys P, Deriemaeker P, Hebbelinck M. Estimation of daily human intake of food flavonoids. Plant Foods Hum Nutr 2007;62:93. [PMID: 17597415]

31. Huang L, Zhang D, Han W, Guo C. High-mobility group box 1 inhibitionstabilizes intestinal permeabilitythrough tight junctionsin experimental acute necrotizing pancreatitis. Inflamm Res 2019;68:677-689. [PMID: 31139836]

32. De Dios I, Ramudo L, Alonso JR, Recio JS, Garcia-Montero AC, Manso MA. CD45 expression on rat acinar cells: involvement in pro-inflammatory cytokine production. FEBS Lett 2005;579:6355–6360. [PMID: 16263122]

33. Zhou C, Saxon A, Zhang K. Human activation-induced cytidinedeaminase is induced by IL-4 and negatively regulated by CD45: implication of CD45, as a Janus kinase phosphatase in antibody diversification. J Immunol 2003;170:1887–1893. [PMID: 12574355]

34. Daniel P, Leśniowski B, Mokrowiecka A, Jasińska A, Pietruczuk M, Małecka-PanasE. Pancreatology 2010; 477–482. [PMID: 20720449]

35. Xue LN, Wang XY, Tan Y, Lin M, Zhang W, Xu KQ. Significance of resistinexpressionin acute pancreatitis. Exp Ther Med 2015;9:1438-1442. [PMID: 25780448]

36. Basios N, Lampropoulos P, Papalois A, Lambropoulou M, Pitiakoudis MK, Kotini A, et al. Apigenin attenuates inflammation in experimentally induces acute pancreatitis – associated lung injury. J Invest Surg 2016; 29:121-7. [PMID: 26631908]

37. Markakis C, Tsaroucha A, Papalois A, Lambropoulou M, Spartalis E, Spartalis T, et al. The role of eugenol in the prevention of acute pancreatitis – induced acute kidney injury: experimental study. HPB Sur 2016. [PMID: 26884642]

38. PapayZE, Kallai-Szabo N, Balogh E, Ludányi K, Klebovich I, Antal I. Controlled Release Oral Delivery of Apigenin Containing Pellets with Antioxidant Activity. Curr Drug Deliv 2017;14:145-154. [PMID: 27264725]

39. Tang D, Chen K, Huang L, Li J. Pharmacokinetic properties and drug interactionsofapigenin, a natural flavone. Expert Opin Drug Metab Toxicol 2017;13:323-330. [PMID: 27766890]

40. Singh P, Mishra SK, Noel S, Sharma S, RathSK. Acute exposure of apigenin induces hepatotoxicity in Swiss mice. PLoS One 2012;7:e31964. [PMID: 22359648]