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Changes of bile secretion and liver lipid peroxydation under influence of glicocortycoids in sheep

Zmiany wydzielania żółci i peroksydacji lipidów w wątrobie owiec pod wpływem glikokortykoidów

SUMMARY

Experiments were performed on 9 sheep divided into three groups: I – control; II – treated i.m. with hydrocortisone (20 mg/kg b wt) for 5 days; III – treated with C plus vit.C (20 mg/kg b wt i.m.). for 5 days. Administration of hydrocortisone decreased bile flow to 6.65 ±1.35 µl/min/kg b wt in 165 min. Addition of vit. C resulted in increase ($p<0.05$) of bile output to mean value 12.059 ±0.937 µl/min/kg b wt. Under stressful conditions increase ($p<0.05$) of biliary concentration and secretion of malondialdehyde (MDA) was observed (max. 0.952 ±0.123 µM/min/kg b wt in 120 min.). Thus, biliary level of this compound seems to be a useful marker of oxidative stress in liver. Exposition to vit. C caused reduction of biliary output of MDA averaged at 0.461 ±0.029 µM/min/kg b wt. Biliary cholesterol (CH) concentration in hydrocortisone-treatment sheep gradually increase to max. 191.75 ±8.31mg/100ml in 105 min. After vit. C administration considerable ($p<0.05$) reduction of biliary level of this compound was observed. On the other hand, elevation of biliary output of CH to highest value 18.965 µM/min/kg b wt in 135 min of experiment was found. In conclusion, administration of ascorbic acids ameliorates glicocorticoids induced lipid peroxydation and cholesterol crystal formation in liver.

Key words: glucocorticoids, malondialdehyde, lipid peroxydation, bile, vitamin C, sheep

INTRODUCTION

Oxidative stress, generated by glucocorticoids (GCs) has been implicated in many hepatobiliary disturbances, which might lead to the bile flow reduction, cholesterol crystal formation and primary biliary cirrhosis [Seckin *et al.* 1997, Hu *et al.* 2000]. Excessive secretion of stress hormones exerts immunosupresive, hyperglycaemic, hypertensive and muscle degradative effects including its atrophy [Hu *et al.* 2000, Eid *et al.* 2003]. Moreover, they caused an imbalance be-

tween free radicals and antioxidant protective system resulted in progress of oxidative stress in many tissues. Numerous results indicate that a high level of plasma GCs may stimulate free radicals formation due to the accumulation of excessive amounts of free fatty acids. One of the most important consequences of the free radicals generation, in such conditions, is lipid peroxydation regarded as a general mechanism whereby reactive oxygen species (ROS) induce tissue damage, especially in liver and heart [Wong *et al.* 1987]. Such a state, among other things, leads to the generation of malondialdehyde (MDA), which is the end product of peroxidative degradation of polyenic fatty acids. Thus, concentration of this compound is used as an index of oxidative stress and directly indicates the extent of lipid peroxydation [Eid *et al.* 2003]. However, a lot of studies focused only on the plasma or tissue level of MDA but no attempt has been made to assess concentration of this compound in bile. Taking into account the fact that oxidative damage of lipids has been implicated in various hepatobiliary disorders it seems to be important to examine changes in the level and secretion of this sensitive marker in bile. In our experimental approach we analysed changes in bile flow and biliary MDA and cholesterol secretion in response to GCs induced oxidative stress in liver of chronically canulated sheep.

It is known that vit.C protects cell components from free radicals-induced damage by quenching various water-soluble radicals, scavenging lipid peroxidation-derived radicals or reducing tocopherol radicals to tocopherol [Stehbens 2003]. Yamamoto *et al.* [1998] observed that during incubation of human plasma under aerobic condition at 37°C ascorbate concentration was markedly reduced without any significant changes in the level of another antioxidants, mainly α-tocopherol. It seems that vitamin C sparing other antioxidants by forming the first line of defence against free radicals and peroxides. Hence, another major purpose of this study was to determine the effect of ascorbic acid, water-soluble antioxidant, on biliary lipid peroxydation generated by administered glicocorticoids.

MATERIALS AND METHODS

Experiments were performed on 9 Polish Lowland sheep, adapted during 4 weeks, weighing between 30–35 kg, 1.5 years of age each. The animals were fed with hay and crushed oats given twice daily. Surgery was performed after withdrawal of food for 48 h and water for 24 h under general anaesthesia induced with 0.5 ml i.m. of Xylazine and i.v. infusion with 2 ml/kg b wt of Vetbutal in physiological solution. During the surgery, the sheep were fitted with a standard T cannula to the duodenum and polyethylene tube to the common bile duct, and were removed gallbladder after cystic duct ligature. Jugular vein catheters (15-G, Venocath) were introduced immediately before the experiments. The cannulated animals were divided into three groups: I – control group; II – treated intramuscularly for 5 days with hydrocortisone (20 mg/kg b wt); III – treated for 5 days with hydrocortisone together with ascorbic acid (20 mg/kg b wt i.m.). The sheep were deprived of food and bile flow was disrupted for 24 hours before experiment. On 5 day of administration of hydrocortisone alone and combined with vit.C in each of experimental groups bile samples were collected during 15-min periods within 3 hours and their volume was measured immediately.

Biliary concentration of MDA was measured by spectrophotometric method [Ledwożyw *et al.* 1986] with some modification. Samples of 0.25 ml bile were mixed with 1.25 ml of trichloracetic acid (TCA) and with 0.75 of thiobarbituric acid and thereafter heating for 20 min in a boiling water bath. After cooling to the room temperature 2 ml of n-butanol was added and the mixture was shaken vigorously for 3 min and centrifuged 10 min at 1500 × g. After transfer of upper n-butanol layer to glass cuvette its absorbance was measured at 532 nm. Concentrations of MDA were read from standard curve obtained by using malondialdehyde bis-dimethylacetal.

The total biliary cholesterol concentration was determined in duplicate by enzymatic method using a diagnostic KIT (Liquick Cor-CHOL 120, Cormay, Poland). The results are based on 2 experiments performed on each of 3 group and are given as mean ± SD. For statistical analysis the Student's *t*-test was used.

RESULTS

During a 3-hour collecting period the bile flow in control group was ranged from 12.375±1.350 µl/min/kg b wt to 17.139 ±2.688 µl/min/kg b wt (Fig. 1). In hydrocortisone-treated sheep the bile flow significantly decreased to minimum value 6.65 ±1.35 µl/min/kg b wt in 165 min and did not exceed 9.5±2.69 µl/min/kg b wt. During combined administration of hydrocortisone and vit. C bile flow markedly increased ($p<0.05$) to the mean value 12.059±0.937 µl/min/kg b wt. but did not reach control values.

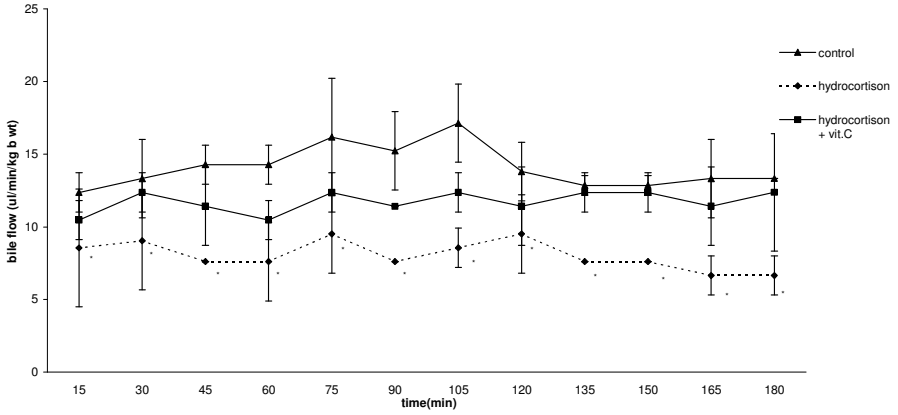

Fig. 1. Bile flow in the control sheep and in sheep treatment with hydrocortisone alone and together with vit. C; means ±SD (n = 3); different from the control at *$p<0.05$

Rys. 1. Wydzielanie żółci u owiec w grupie kontrolnej oraz po podaniu hydrokortyzonu oraz po łącznym podaniu hydrokortyzonu i wit. C; Wyniki zostały przedstawione jako średnia ±SD (n = 3); *różnica istotna statystycznie w porównaniu z kontrolą ($p<0,05$)

As shown in Tab. 1 there was a significant increase of biliary MDA concentration in hydrocortisone–treated group of animals in comparison to control group. In the second experimental group an increase in MDA concentration due to hydrocortisone treatment was markedly reduced by vit. C, but still maintained under control values.

Table 1. Biliary concentration of malondialdehyde (MDA) (μM/ml); means ±SD (n = 3); different from the control, at *p<0.05
Tabela 1. Stężenie aldehydu dwumalonowego (μM/ml) w żółci; wartości stanowią średnią ±SD (n = 3); * różnica istotna statystycznie w porównaniu z kontrolą (p<0,05)

	Time (min)											
	15	30	45	60	75	90	105	120	135	150	165	180
Control Group	35.28 ±2.375	27.62 ±1.385	34.30 ±4.384	29.00 ±3.676	27.68 ±1.810	29.00 ±4.242	30.20 ±4.808	35.96 ±1.414	22.88 ±0.113	22.40 ±4.525	27.08 ±3.563	28.38 ±4.836
Hydrocortisone-treated group	139.825* ±54.694	106.455* ±25.321	71.78* ±6.462	90.475* ±0.742	81.935* ±18.363	93.625* ±39.845	86.135* ±28.263	127.225* ±32.915	95.305* ±49.150	95.375* ±48.260	122.50* ±34.648	113.715 ±23.709
Hydrocortisone plus vit. C –treated group	37.10 ±2.474	47.45* ±1.202	49.70 ±15.344	77.00* ±29.698	56.35 ±12.869	64.925* ±12.621	49.525 ±17.076	67.20 ±31.183	49.525* ±7.672	57.59* ±16.065	59.15* ±13.364	50.05 ±18.314

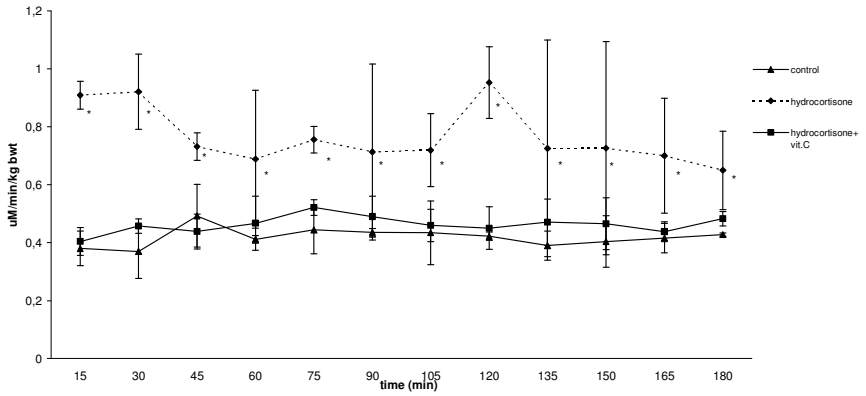

Fig. 2. Biliary malonyldialdehyde secretion in the control sheep and treatment with hydrocortisone alone and together with vit. C; means ±SD (n = 3); different from the control and first experimental group, at *p<0.05

Rys. 2. Wydzielanie aldehydu dwumalonowego do żółci owiec w grupie kontrolnej oraz po podaniu hydrokortyzonu oraz po łącznym podaniu hydrokortyzonu i wit. C; wyniki zostały przedstawione jako średnia ±SD (n = 3); *różnica istotna statystycznie w porównaniu z kontrolą i pierwszą grupą doświadczalną (p<0,05)

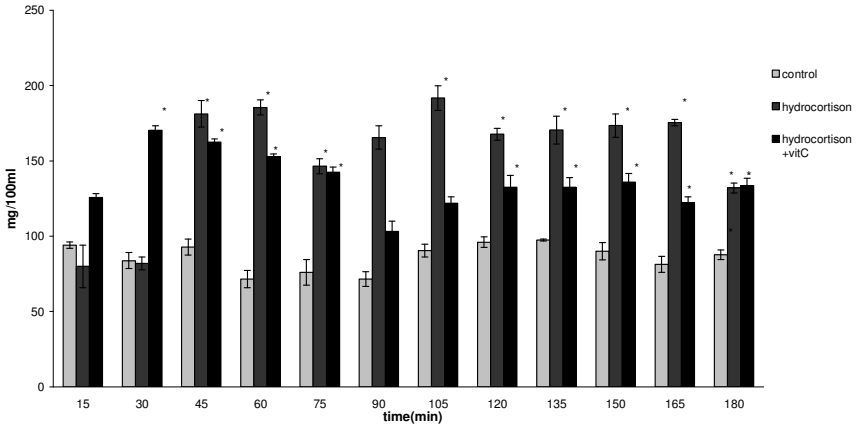

Fig. 3. Biliary concentration of cholesterol in the control sheep and in sheep treatment with hydrocortisone alone and together with vit. C; means ±SD (n = 3); different from the control, at *p<0.05

Rys. 3. Stężenie cholesterolu w żółci u owiec w grupie kontrolnej oraz po podaniu hydrokortyzonu oraz po łącznym podaniu hydrokortyzonu i wit.C; Wyniki zostały przedstawione jako średnia ± SD (n = 3); *różnica istotna statystycznie w porównaniu kontrolą (p<0,05)

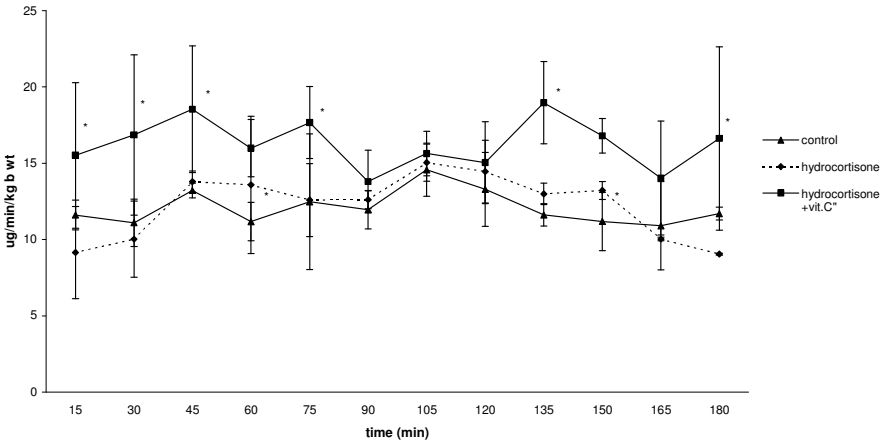

Fig. 4. Biliary secretion of cholesterol in the control sheep and in sheep treatment with hydrocortisone alone and together with vit. C; means ±SD (n = 3); different from the control, at *p<0,05

Rys. 4. Wydzielanie cholesterolu do żółci u owiec w grupie kontrolnej oraz po podaniu hydrokortyzonu oraz po łącznym podaniu hydrokortyzonu i wit. C; wyniki zostały przedstawione jako średnia ± SD (n = 3); *różnica istotna statystycznie w porównaniu z kontrolą (p<0,05)

Administration of hydrocortisone alone substantially increased biliary secretion of MDA during all experimental period with maximum value 0.952 ±0.123 µM/min/kg b wt in 120 minutes (Fig. 2). The same infusion, but with exposition to vit. C resulted in significant reduction of biliary output of MDA averaged at 0.461 ±0.029 µM/min/kg b wt.

After thirty minutes of bile collection, concentration of cholesterol in hydrocortisone-treatment animals gradually increased to maximum value 191.75 ±8.31mg/100ml in 105 min. (Fig. 3). In the second experimental group considerable (p<0,05) reduction of biliary level of cholesterol was observed, especially in the last hour of experiment when the value of cholesterol ranged between 122.25 ±3.89 mg/100 ml and 133.5 ±4.94 mg/100 ml. As shown in Fig. 4 there was no significant difference in biliary output of this compound in the first experimental group. Administration of vit. C let to increase of cholesterol secretion above control results with the highest value reached 18.965 µM/min/kg b wt in 135 min of experimental period.

DISCUSSION

Glicocorticoids treatment develops various hepatobiliary disorders due to the formation of free radicals, which cause extensive lipid peroxydation in hepatic membranes. These pathological changes lead to alterations in membrane potential and fluidity and finally result in changes of bile production and especially its secretion. Our results obtained in the first group of animals directly show that prolonged administration of hydrocortisone significantly reduced bile flow during all collecting period in comparison to

control animals. It should be taken into account that canalicular transport of the main constituents of bile, which directly created driving force for bile flow, mainly bile salts, sodium, glutathione and HCO_3^-, depends on a series of protein carriers: canalicular ATP-dependent bile salt transporter (cBST), Na^+-K^+-ATPase, multidrug resistance protein 2 – MRP2, exchangeable pomp for HCO^-_3 [Kullak-Ublick *et al.* 2000, Wójcik *et al.* 2003]. Dysfunction of any above mentioned protein carriers might results in cholestasis. One of the ways to disturb the activity of protein carriers is cytotoxic aldehydes and lipid peroxides, which can inactivate proteins and also inhibit their synthesis [Sreejayan 1999]. In our study concomitant with reduction of bile flow under stressful condition, dramatic elevation of malondialdehyde levels in bile was found, directly indicated intensive peroxidative damage of lipids. Therefore, a considerable ($p<0.05$) increase of biliary output of this compound was found in hydrocortisone-treated animals. Combined administration of vitamin C and hydrocortisone in the second experimental group resulted in significant elevation of bile flow connected with reduction of biliary MDA secretion. Taking into account the results obtained in both our experimental groups, administration of ascorbic acid greatly limited hydrocortisone-associated cholestasis and lipid alterations induced by ROS. On the other hand, the produced endogenously ascorbic acid seems to be insufficient to quench reactive oxygen species generated by hydrocortisone treatment.

One of the most important functions of hepatic bile formation and bile flow is excretion of cholesterol from the body, by three different ways. First of all it is used as a substrate for bile acids formation; furthermore, is removed, as the free water-insoluble compound and by formation of mixed micelles with bile salts [Engelking *et al.* 1989, Oude Elferink *et al.* 1995, Gilat *et al.* 1996, Koopen *et al.* 1998]. Under stressful conditions generated by hydrocortisone treatment in the first group of animals, concomitant with elevation of biliary MDA level, a marked increase of cholesterol concentration was observed. Moreover, there were no significant changes in biliary cholesterol output in comparison to control value. Hence, our results are in concert with the measurements of biliary MDA levels, which were higher in patients with cholesterol gallstones in comparison to stone free patients. Moreover, *in vitro* results show that generation of lipid peroxidation products significantly reduced the cholesterol crystal formation time, indicating that peroxide damage of lipids may play a role in cholesterol gallstone diseases [Eder *et al.* 1996, Sreejayan *et al.* 1999]. It has been pointed out that ascorbic acid is necessary for the activation of hepatic cholesterol-7α-hydroxylase [Jayachandran *et al.* 1996]. Deficiency of vit. C causes depression of this enzyme, which is a rate-limiting step in cholesterol transformation to bile acids. In this light, a decrease of biliary level of cholesterol and its elevated secretion obtained in the present study in animals with ascorbic acid administration may be attributed to the activation of 7α-hydroxylase by this vitamin.

CONCLUSIONS

1. The level of MDA in bile reflects the range of lipid peroxydation in liver generated by oxidative stress induced e.g. by GC.

2. Lipid peroxydation products disturbed the process of canallicular bile formation, which led to drop in the volume of secreted bile.

3. Administration of ascorbic acid seems to protect the liver against ROS generated by glicocorticoids treatments.

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STRESZCZENIE

Doświadczenie przeprowadzono na 9 owcach rasy PON, podzielonych na trzy grupy: I – kontrolna, II – grupa otrzymująca przez 5 dni hydrokortyzon (20 mg/kg m.c. i.m.), II – grupa otrzymująca łącznie z hydrokortyzonem witaminę C (20 mg/kg m.c. i.m.).W porównaniu z kontrolą podanie hydrokortyzonu spowodowało znaczny ($p<0,05$) spadek wydzielania żółci do 6,65 ±1,35 µl/min/kg m.c. w 165 min. Ekspozycja zwierząt na witaminę C znosiła hamujący wpływ hydrokortyzonu, dając w rezultacie wzrost wydzielania żółci, osiągający średnie wartości 12,059 ±0,937 µl/min/kg m.c. W warunkach indukowanego hydrokortyzonem stresu obserwowano istotny ($p<0,05$) wzrost zarówno stężenia, jak i wydzielania MDA do żółci (max. 0,952 ±0,123 µM/min/kg m.c. w 120 min.). Dodatek wit. C spowodował spadek sekrecji MDA do średniej wartość 0,461 ±0,029 µM/min/kg m.c. Stężenie cholesterolu wzrastało stopniowo do 191,75 ±8,31mg/100ml w 105 min w żółci owiec poddanych działaniu hydrokortyzonu. Jednocześnie z istotnym ($p<0,05$) spadkiem poziom cholesterolu po podaniu wit. C obserwowano również wzrost jego wydzielanie do żółci, osiągający najwyższą wartość 18,965 µM/min/kg m.c. w 135. min doświadczenia. Zarówno zmiany poziomu aldehydu dwumalonowego, jak i cholesterolu w żółci pod wpływem podawanego kwasu askorbinowego wskazują, iż jest on czynnikiem ochraniającym wątrobę w warunkach stresu oksydatywnego wywołanego podawaniem hydrokortyzonu.

Słowa kluczowe: glikokortkoidy, peroksydacja lipidów, aldehyd dwumalonowy, żółć, witamina C