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Comparison of S-Adenosyl-l-methionine and N-Acetylcysteine Protective Effects on Acetaminophen Hepatic Toxicity

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d) Abbreviations

ALT alanine aminotransferase

APAP acetaminophen, [4-hydroxyacetanilide]

GSH glutathione

GSSG glutathione disulfide

4-HNE 4-hydroxynonenal

NAC n-acetylcysteine

SAMe S-adenosyl-l-methionine

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ABSTRACT

Nutraceuticals are widely used by the general public but very little information is available regarding the effects of nutritional agents on drug toxicity. Excessive doses of acetaminophen (APAP, 4-hydroxyacetanilide) induce hepatic centrilobular necrosis. The naturally occurring substance S-adenosyl-l-methionine (SAMe) has been reported to reduce the hepatic toxicity of acetaminophen (APAP). The present study was designed to investigate the hepatoprotective effects of SAMe in comparison to the clinically used antidote, n-acetylcysteine (NAC). Male C57BL/6 mice were injected intraperitoneal (ip) with an equimolar dose (1.25 mmol/kg) of either SAMe or NAC just prior to APAP and the groups were denoted SAMe+APAP and NAC+APAP, respectively. Mice were immediately injected (ip) with 300 mg/kg APAP and hepatotoxicity was evaluated after 4 h. SAMe was more hepatoprotective than NAC at a dose of 1.25 mmol/kg as liver weight was unchanged by APAP injection in the SAMe+APAP group while liver weight was increased in the NAC+APAP group. SAMe was more hepatoprotective for APAP toxicity than NAC since ALT levels were lower in the SAMe+APAP. Pretreatment with SAMe maintained total hepatic glutathione (GSH) levels higher than NAC pretreatment prior to APAP, although total hepatic GSH levels were lower in the SAMe+APAP and NAC+APAP groups than the vehicle (VEH) control values. Oxidative stress was less extensive in the SAMe+APAP group when compared to the APAP treated mice as indicated by Western blots for protein carbonyls and 4-hydroxynonenal (4-HNE) adducted proteins. In summary, SAMe reduced APAP toxicity and was more potent than NAC in reducing APAP hepatotoxicity.

INTRODUCTION

Acetaminophen (APAP) or N-(4-hydroxyphenyl acetamide), is a commonly used over the counter medication contained in more than 125 products within the United States. APAP is widely used as an antipyretic and analgesic agent. Acetaminophen (APAP) in excessive doses can induce hepatic centrilobular necrosis (Davidson and Eastham, 1966; McJunkin et al., 1976). Acute administration in animals can induce similar damage to the liver within the centrilobular region (Mitchell et al., 1973 a,b; Jollow et al., 1973).

APAP hepatotoxicity requires cytochrome P450 mediated formation of the toxic metabolite, N-acetyl-p-benzoquinoneimine (NAPQI) (Mitchell et al., 1973 b). Glutathione (GSH) is crucial in detoxification of NAPQI. However, when excessive amounts of APAP are ingested, the formation of NAPQI is greater than the supply of GSH resulting in depletion of intracellular stores of GSH and hepatic damage.

S-adenosyl-L-methionine (SAMe) has been shown to reduce APAP hepatotoxicity by our laboratory (Valentovic et al., 2004) as well as others (Stramentinoli et al., 1979; Bray et al., 1992; Carrasco et al., 2000; Song et al., 2004). SAMe administration just prior to APAP treatment greatly diminished the extent of centrilobular necrosis in C57BL/6 male mice when compared to animals given APAP (Valentovic et al., 2004). SAMe has also shown beneficial effects in reducing and reversing hepatic damage induced by alcohol in humans (Lieber, 2002) and in intrahepatic cholestasis (Frezza et al., 1990).

(SAMe) is a naturally occurring substance present in plasma and most tissues. SAMe is commercially available and marketed worldwide as a nutritional supplement that is readily available in vitamin and health food stores. SAMe is the principal methyl donor within the body, for transmethylation reactions. The major site of transmethylation reactions is the liver. SAMe is also a vital precursor for the transsulfuration pathway. The final product of the transsulfuration pathway is glutathione. SAMe is metabolized to S-adenosylhomocysteine (SAH) and then to homocysteine by a hydrolase enzyme. Homocysteine is converted to cystathionine by cystathione β-synthase and cystathionine is subsequently converted to cysteine, a precursor for GSH, by γ-cystathionase (Lu, 1998). Cysteine is subsequently converted by glutamate-cysteine ligase (EC 6.3.2.2), the rate limiting enzyme in GSH synthesis, to γ-glutamylcysteine which is finally converted by GSH synthetase to GSH.

N-acetylcysteine (NAC) is the current clinical treatment for APAP overdose. Hepatic GSH depletion is a **critical** component in the development of hepatic necrosis resulting in an increase in NAPQI (Nelson and Bruschi, 2003) which reacts with protein macromolecules and causes cell damage. NAC reduces APAP hepatic toxicity by increasing GSH levels and maintaining thiol status (Corcoran and Wong, 1986). NAC reduces APAP hepatic damage by providing cysteine as a precursor for GSH (Lauterburg et al., 1983; Corcoran et al., 1985a, b).

As mentioned above, NAC is the standard clinical antidote for APAP hepatic overdose. Evaluation of a potential antidote, such as SAMe, would necessitate a comparison to NAC. The current study was designed to evaluate the protective effect of SAMe on APAP hepatic toxicity in comparison to the current antidote, NAC. The potential beneficial effect of SAMe to reduce

APAP hepatic toxicity can be properly evaluated only after comparison to the standard antidote of NAC. Comparisons were made of the protective effect of an equimillimole dose of NAC and SAMe on APAP hepatic toxicity to evaluate the potency of SAMe. The present study further explored the potential property of SAMe to modify APAP mediated oxidative stress.

MATERIALS AND METHODS

Materials S-adenosyl-l-methionine (SAMe) was used in all studies as the toluenesulfonate salt (Sigma Chemical Co., St. Louis, Mo.). Malondialdehyde was purchased from Aldrich Chemical Company (St. Louis, Mo.). Glutathione, NADPH, 2-vinylpyridine, thiobarbituric acid and all other reagents were purchased from Sigma Chemical Company. The ALT reagent kit (TR-71021) was purchased from Thermo Electron Corporation (Louisville, CO).

Animals All studies were conducted in 4-5 week old male C57BL/6 mice weighing 16-20 grams which were obtained from Hilltop Lab Animals Inc. (Scottsdale, PA). The protocol was approved by the University Committee on Animal Care and Use. Mice were housed in a facility accredited by the American Association for Accreditation of Laboratory Animal Care. Mice were maintained under a controlled ambient temperature (21-23^{o}C), humidity (40-55%) and 12 h light cycle (lights on 0600-1800 h). Mice were acclimated for 7 days prior to initiation of any procedures. Animals had free access to water and Purina Rodent Chow prior to initiation of any treatment. However, mice were fasted prior to treatment with APAP as indicated below.

SAMe and NAC treatments just prior to APAP Mice were randomly divided into the following groups: vehicle treated (VEH); APAP treated (APAP), SAMe treated (SAMe), SAMe pretreated plus APAP (SAMe+APAP), NAC (NAC), NAC pretreated plus APAP (NAC+APAP). Each group contained 5-10 different mice/group. Mice were fasted for 16 h prior to injection of APAP beginning with removal of food at 1700 h the previous day. Mice in the SAMe and SAMe+APAP groups were injected intraperitoneal (ip) with 1.25 mmol/kg (5 ml/kg) SAMe at 0900 h. Mice in the NAC and NAC+APAP groups were injected (ip) with 1.25 mmol/kg (5

ml/kg adjusted to pH 7) NAC. Immediately following treatment with SAMe or NAC, mice in the APAP, SAMe+APAP and NAC+APAP groups were injected, ip, with 300 mg/kg APAP (15 ml/kg in warm water). The VEH group was injected, ip, with water at an equivalent volume (15 ml/kg). Mice were anesthetized with carbon dioxide 4 h after APAP administration. Blood was collected by cardiac puncture using heparin rinsed 1 ml syringes (20 gauge needles) for determination of alanine aminotransferase (ALT) activity, a biomarker of liver injury. The livers were isolated, collected on ice, rinsed in ice cold Krebs buffer, blotted and weighed.

NAC high dose treatment given just prior to APAP Studies were done with a higher dose of NAC reported by other investigators to provide complete protection (Corcoran et al., 1985a, b; James et al., 2003) in order to validate our model. Mice were divided into the following groups: vehicle treated (VEH); APAP treated (APAP), NAC (NAC-H) and NAC pretreated plus APAP (NAC-H+APAP). Each group contained 5-10 different mice/group. Animals were fasted overnight for 16 h. Mice were injected with 7.35 mmol/kg NAC (5 ml/kg in water adjusted to pH 7). Immediately after injection of NAC-H, mice were injected, ip, with 300 mg/kg APAP. Mice were anesthetized with carbon dioxide 4 h after APAP. Blood was collected via cardiac puncture. The liver was quickly excised and rinsed in ice cold buffer.

Serum enzyme assay Blood was collected for measurement of ALT levels. Plasma was collected following centrifugation of blood for 15 min at 9000xg in a microcentrifuge. Plasma was stored at 4^{o}C until ALT levels were measured. ALT levels were measured using an enzymatic kit (TR-71021) obtained from Thermo Electron Corporation (Louisville, CO).

Glutathione determination Tissues (200 mg) were homogenized in 500 µl 0.5% sulfosalicylic acid and adjusted to a 1 ml volume. Total glutathione was determined using a glutathione reductase and NADPH coupled reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (Griffith, 1980; Andersen, 1985) and expressed as nmol/g tissue. Glutathione disulfide (GSSG) was measured following 2-vinylpyridine derivatization (Griffith, 1980) and expressed as nmol/g tissue.

Lipid peroxidation Liver (200 mg) was homogenized in 1 ml Krebs buffer and the homogenizer probe was rinsed with an additional 1 ml of Krebs buffer. Lipid peroxidation was measured as described previously (Valentovic et al., 2002). The amount of malondialdehyde (MDA) was calculated based on a standard curve (range 1-40 nmol) using MDA (Aldrich, St. Louis, Mo) and expressed as umol MDA/mg protein.

4-Hydroxynonenal (4-HNE) Adducts Western blot was used to analyze for the presence of 4-HNE protein adducts. For each sample 150 µg protein were analyzed following a boiling period of 5 min. Samples and standards were run on polyacrylamide gel (12.5 % acrylamide). The gel was then transferred to a nitrocellulose membrane. To verify efficiency of transfer, the membrane was placed in Ponceau S stain for visualization. The membrane was then rinsed with H_2O at room temperature under constant shaking to remove stain. Nonspecific protein binding was blocked with milk at room temperature under constant shaking for 1 hr. Rabbit polyclonal antibody to (E)-4-Hydroxynonenal (anti-HNE PAb) (1:1000; Alexis Biochemicals, ALX-210-767) was added and incubated overnight at 4°C under constant shaking. The membrane was then rinsed three times in TBST. The secondary antibody, goat anti-rabbit linked with horseradish peroxidase (1:3000) in blocking buffer was added and incubated at room temperature

under constant shaking for 90 min. The membrane was rinsed three times in TBST and developed using enhanced chemiluminescent substrate for visualization of 4HNE-adducted proteins.

Protein carbonyl (OxyBlot) Protein carbonyls were evaluated to assess whether SAMe reduced APAP mediated oxidative stress relative to NAC. Hepatic tissue was weighed, homogenized in 1 ml Krebs buffer pH 7.4 and the homogenizer probe was rinsed with 1 ml Krebs buffer. The homogenate was adjusted to 2 ml. An aliquot (200 µl) was used for protein determination using a Coomasie Blue spectrophotometric method (Valentovic et al., 1999). The appearance of protein carbonyls was measured using an Protein Oxidation OxyBlot kit (Chemicon). The principle of the method is that modification of proteins by addition of carbonyl side chains due to oxidative stress are derivatized to 2,4-dinitirophenylhydrazone (DNP) following addition of 2,4-dinitrophenylhydrazine (DNPH). The antibody is specific for the DNP moiety on a protein.

Tissue samples were adjusted to ensure equivalent protein and volumes were added to gels. Samples and standards were run on polyacrylamide gel (12.5 % acrylamide). The gel was then transferred to a nitrocellulose membrane. To verify efficiency of transfer, the membrane was placed in Ponceau S stain for visualization. Ponceau S staining was used on all gels to ensure protein additions were comparable between all samples. The protein carbonyls were run with DNP-derivatized molecular weight standards.

Histological preparation A segment of liver was fixed in 15 ml neutral buffered formalin solution. The tissues were embedded in Paraplast and processed. The tissue was sectioned into

approximately 4μm thickness and stained with hematoxylin and eosin (H&E). The tissues were viewed with a Nikon light microscope for evidence of hepatic necrosis.

Statistical analysis Values represent Mean ± SEM with n=5-10 animals/group. Differences between groups were analyzed using an analysis of variance (ANOVA) followed by a Tukey test (Sigma Stat, SPSS Inc. Chicago, IL). All statistical analyses were conducted using a 95% confidence interval.

RESULTS

Liver Weight, Plasma ALT and liver histological evaluation. Body weights were comparable between all treatment groups (Table 1). APAP increased liver weight within 4 h when compared to the VEH group (Table 1). Treatment with NAC or SAMe had no impact on liver weight as values were comparable to the VEH group. Liver weight was not elevated in the SAMe+APAP group following APAP injection (Table 1) as liver weight was similar to the VEH and SAMe groups. Treatment with an equimolar dose of NAC just prior to APAP injection (NAC+APAP) resulted in elevated liver weight when compared to the VEH and NAC groups. Liver weight values were the similar between APAP and NAC+APAP groups. These findings indicated that when comparing equimolar (1.25 mmol/kg) treatment of SAMe and NAC just prior to APAP injection, SAMe provided better protection than NAC. Administration of a higher dose (7.35 mmol/kg) NAC resulted in the best protection of liver weight as the NAC-H+APAP group was similar to the NAC-H group. Experiments were not conducted with an equimolar 7.35 mmol/kg dose of SAMe due to solubility issues and the cost of SAMe.

Normal morphology was observed in the VEH, SAMe and NAC groups following light microscopic examination of H&E stained hepatic tissues (Figures 1A,B and 2A, B). Examination of the VEH group indicated 10/10 mice had no evidence of hepatic necrosis. Examination of the SAMe and NAC group showed 10/10 and 10/10 mice, respectively, had <5% cells with hepatic necrosis. Extensive zonal centrilobular necrosis was evident in the APAP group (Figure 1C). Examination of APAP treated tissue indicated 5/5 animals had marked centrilobular necrosis as indicated by (>40% necrotic cells). The extent of centrilobular necrosis was less severe in the SAMe+APAP group (Figure 1D) when compared to the APAP treated mice. The SAMe+APAP

group had less centrilobular necrosis as 1/5 had mild degeneration (10-25% necrotic cells), 3/5 had moderate lesions (25-40% necrotic cells) and only 1/5 with >40% necrotic cells. An equimolar dose of 1.25 mmol/kg NAC given just prior to APAP resulted in some protection but necrotic lesions were apparent around the centrilobular region (Figure 2C). The NAC+APAP group exhibited 3/5 with marked centrilobular necrosis (>40% necrotic cells) and 2/5 with moderate lesions (25-40% necrotic cells). Thus, the protection by 1.25 mmol/kg NAC (NAC+APAP) was not as extensive as SAMe+APAP. As a positive control, treatment with 7.35 mmol/kg NAC (NAC-H) just prior to APAP resulted in the best protection as the NAC-H+APAP mice exhibited normal structure and 5/5 mice had very moderate degeneration (10-25% necrotic cells) of the centrilobular region (Figure 2D).

ALT levels were evaluated in plasma collected 4 h after vehicle or APAP injection. ALT values were similar in the VEH, SAMe and NAC groups (Table 2) suggesting normal hepatic function. APAP administration resulted in the highest increase in ALT levels which were approximately 100 fold higher than VEH values. The 1.25 mmol/kg NAC dose given just prior to APAP did not reduce ALT levels in the NAC+APAP group when compared to the APAP group. Treatment with 1.25 mmol/kg SAMe did provide partial protection from APAP toxicity as ALT levels were reduced in the SAMe+APAP group treatment relative to the APAP and NAC+APAP group providing support for the conclusion that SAMe does reduce APAP hepatic toxicity.

Glutathione and glutathione disulfide (GSSG) following APAP treatment. Total hepatic GSH levels were comparable between VEH, SAMe and NAC groups (Figures 3-6) which suggests that total hepatic GSH levels were not increased by SAMe or NAC treatment. Total

hepatic GSH was extensively diminished within 4 h after APAP injection (Figures 3-6) as GSH levels were decreased over 90% in the APAP group when compared to VEH, SAMe or NAC values. SAMe administration just prior to APAP partially reversed the depletion of total hepatic GSH (Figure 3). GSH levels were higher in the SAMe+APAP group than in the APAP group but were lower than the SAMe and VEH groups suggesting that the 1.25 mmol/kg dose of SAMe was able to partially maintain hepatic thiol levels in APAP treated mice.

Total hepatic GSH levels in the 1.25 mmol/kg NAC+APAP group were comparable to mice treated with APAP (Figure 4). A comparison of hepatic GSH values between groups showed that SAMe+APAP were higher than NAC+APAP values (Figure 5) and that SAMe+APAP was able to provide a higher GSH level. These results suggest that at the 1.25 mmol/kg dose, SAMe was more potent than NAC at protecting the liver from APAP mediated GSH depletion (Figure 5).

As a positive control, experiments were conducted to validate NAC attenuation of APAP toxicity. Treatment with the higher dose (7.35 mmol/kg) of NAC (NAC-H) just prior to APAP (NAC-H+APAP) maintained total hepatic GSH at a level comparable to VEH and NAC groups (Figure 6) confirming that 7.35 mmol/kg NAC was the most effective dose at preventing APAP mediated hepatic toxicity. These results were also consistent with the normal morphology observed by light microscopy of hepatic tissues.

APAP mediated oxidative stress resulted in a higher percentage of hepatic GSSG (Figures 7-9) when measured 4 h after APAP injection. The percent GSSG was not affected by equimolar dose of either SAMe or NAC as the levels were similar between the VEH, SAMe and NAC groups

(Figure 7 and 8). Pretreatment with 1.25 mmol/kg SAMe (SAMe+APAP) or NAC (NAC+APAP) did not alter the percent of GSSG following APAP injection (Figures 7-8). Although not statistically different, the NAC+APAP group had a trend of higher GSSG than the APAP group while the levels had a trend of being lower in the SAMe+APAP group. Treatment with a high dose of NAC (NAC-H) was able to reduce oxidative stress as the percent of GSSG in the NAC-H+APAP group was comparable to VEH and NAC-H values (Figure 9).

Western Blot analysis of protein carbonyls and 4-HNE The profile for protein carbonyls was similar between VEH and SAMe groups (Figure 10) as well as VEH and NAC groups (Figure 11) suggesting that the extent of protein carbonyl formation was similar between VEH, SAMe and NAC groups. Western blot analysis showed protein carbonyls were elevated 4 h after APAP treatment consistent with an induction of oxidative stress. Increased protein carbonyl positive staining was noted between 37 kDa and 82 kDa when compared to VEH, SAMe and SAMe+APAP groups (Figure 10). These results suggest that SAMe reduces APAP mediated oxidative stress in hepatic tissue. NAC treatment at an equimolar dosage of 1.25 mmol/kg just prior to APAP (NAC+APAP) did not reduce protein carbonyl formation in hepatic tissue (Figure 11). The lack of a protective effect on protein carbonyls in the NAC+APAP group was consistent with the greater decline in GSH observed in the NAC+APAP compared to the SAMe+APAP group (Figure 5). Western blots for 4-HNE indicated an increase in 4-HNE antibody positively stained bands following APAP treatment between 37-115 kDa (Figures 12 and 13). The SAMe+APAP group exhibited less band intensity than the APAP treated liver tissue at the 37-115 kDa bands. The NAC+APAP group was comparable to the APAP group for the profile of bands between 64-115 kDa which were not as apparent in the VEH and NAC groups. These

results were in agreement with the hepatic GSH measurements and the protein carbonyl western blots.

Discussion

The objective of the present study was to compare the protective effects of NAC and SAMe for APAP toxicity. The present study showed that SAMe was effective at a lower dose than NAC when administering an equivalent mmole dose. These results suggest that first SAMe is effective in reducing APAP hepatotoxicity and second, that SAMe is more potent than NAC in reducing hepatic damage in the mouse model.

NAC is the drug of choice for emergency room treatment of APAP overdose (Kerr et al., 2005). NAC diminishes hepatic APAP toxicity by increasing glutathione levels and reducing the extent of hepatic glutathione depletion (Lauterburg et al., 1983; Corcoran et al., 1985 a,b). Glutathione is critical for conjugation with the reactive metabolite of APAP and treatment with NAC allows generation of sufficient levels of glutathione to react with the toxic intermediate, NAPQI and prevent hepatic necrosis (Mitchell et al., 1974; Lauterburg et al., 1983; Corcoran et al., 1985a, b). When hepatic glutathione is depleted by APAP, NAPQI induces cell damage by arylation of hepatic proteins.

Although NAC is the drug of choice for APAP overdose, it is associated with a variety of adverse effects (Kao et al., 2003). NAC is also not entirely protective for APAP toxicity. Therefore, other agents should be examined for their effectiveness. Beneficial protective effects for APAP hepatotoxicity have been reported for clofibrate (Chen et al., 2002), ribose cysteine (Lucas et al., 2000; Lucas Slitt et al., 2005), L-cysteine glutathione mixed disulfide (Berkeley et al., 2003) and 2(RS)-n-propylthiazolidine-4(R)-carboxylic acid (Srinivasan et al., 2003). The mechanism of APAP protection may be different between these agents and remains to be

completely identified for clofibrate. Ribose cysteine , L-cysteine glutathione disulfide, ribose cysteine and 2(RS)-n-propylthiazolidine-4(R)-carboxylic acid act as a provider of cysteine. The cysteine would then provide a sufficient cysteine to the liver to increase GSH synthesis and reduce hepatic GSH depletion following APAP overdose.

SAMe has been previously reported by our laboratory (Valentovic et al., 2004) as well as others (Stramentinoli et al., 1979; Bray et al., 1992; Carrasco et al., 2000) to reduce APAP toxicity. SAMe is a substrate for the transsulfuration pathway which converts homocysteine ultimately to glutathione (Lu, 1998). A potential mechanism for SAMe reduction of APAP hepatotoxicity may involve maintenance of hepatic glutathione levels, which would diminish hepatic glutathione depletion and NAPQI interaction with cellular components to induce necrosis.

In our studies hepatic glutathione levels were higher in the SAMe+APAP group when compared to the APAP treated mice suggesting that SAMe was able to reduce the extent of APAP mediated glutathione depletion. The SAMe+APAP group also had higher hepatic glutathione levels than the NAC+APAP group when comparing the equimolar dose (1.25 mmol/kg SAMe and NAC) suggesting better potency for SAMe. Glutathione levels are essential in preventing APAP toxicity as the NAC-H+APAP group treated with 7.35 mmol/kg NAC maintained hepatic glutathione levels after APAP treatment at a level comparable to VEH and NAC-H levels.

Oxidative stress has been implicated to have a role in APAP toxicity. 4-HNE is an aldehyde that is generated during lipid peroxidation which can impair cellular function (Hartley et al., 1999). 4-HNE can attach to cellular proteins by a 1,4-Michael addition to certain amino acids such as

cysteine and lysine (Esterbauer et al., 1991). 4-HNE positively stained bands between 37-115 kDa were apparent with 4 h following APAP treatment (Figures 12-13). Oxidative stress was reduced by SAMe pretreatment as the SAMe+APAP group had less 4-HNE (Figure 12) and les protein carbonyl (Figure 10) positively stained bands when compared to the APAP groups.

SAMe is present in many tissues in the body and acts as a principal methyl donor for transmethylation reactions especially within the liver. Transmethylation reactions are essential in maintaining normal cell function as SAMe is the methyl donor for transmethylation of phospholipids, proteins, nucleic acids and ultimately DNA (Mato et al., 1997; Lieber and Packer, 2002). Very little is known regarding the effect of exposure to toxicants and alterations in the transmethylation pathway. Imbalances in SAMe and the transmethylation byproduct, S-adenosylhomocysteine (SAH) do occur in chronic alcohol models (Lieber, 2002; Lu and Mato, 2005). An imbalance in the relative ratio of SAMe:SAH can lead to impaired transmethylation reactions (Lu and Mato, 2005) which would be predicted to deter tissue repair and cell function. Further studies are needed to determine whether APAP alters the balance of SAMe:SAH and transmethylation reactions as part of its mechanism of toxicity.

In summary, our study is the first to compare SAMe with NAC on effectiveness as an antidote for APAP toxicity. Equimolar administration of 1.25 mmol/kg SAMe or NAC just prior to APAP injection showed SAMe was more effective than NAC in reducing APAP hepatic toxicity. NAC administered at a higher dose of 7.35 mmol/kg provided the best protection of APAP toxicity. This study was also the first to provide a comparison of APAP hepatic toxicity when SAMe and

NAC were administered just prior to APAP. These results suggest that SAMe, at least in our model, has some potential as an antidote for APAP toxicity.

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LEGENDS FOR FIGURES

Figure 1. Microscopic examination of hepatic tissue following APAP treatment. Figures are H&E stained hepatic tissue collected 4 h after ip injection of 300 mg/kg acetaminophen (APAP) from male C57BL/6 mice. In some groups animals were injected (ip) with 1.25 mmol/kg SAMe just prior to APAP injection. The slides are representative of all animals in the specified group. Normal morphology was observed in: vehicle (VEH, panel A) and SAMe (panel B) treated tissue. Centrilobular necrosis was evident in the APAP (panel C) group. SAMe administration just prior to APAP (SAMe+APAP) group had less extensive necrosis in the centrilobular region (panel D). All slides are H&E stained at 10 μm slice and 200x magnification.

Figure 2. The effect of NAC on APAP treatment. Hepatic tissue was collected 4 h after ip injection of 300 mg/kg acetaminophen (APAP) from male C57BL/6 mice. In some groups animals were injected (ip) with 1.25 or 7.35 mmol/kg n-actylcysteine (NAC) just prior to APAP injection. Normal morphology was observed in: NAC (panel A) treated tissue. Centrilobular necrosis was evident in the APAP (panel B) group. NAC at a dose of 1.25 mmol/kg just prior to APAP (NAC+APAP) group (Panel C) was associated with some centrilobular necrosis. Injection of 1200 mg/kg, 7.35 mmol/kg NAC (NAC-H+APAP) prevented APAP mediated centrilobular necrosis (panel D). Tissues were cut at a 10 μm thickness and stained with H&E and viewed at 200x magnification.

Figure 3. The Effect of SAMe on Total Hepatic Glutathione (GSH) Levels following APAP injection in C57BL/6 mice. Animals were randomly divided into vehicle (VEH), acetaminophen treated (APAP), SAMe (1.25 mmol/kg) treated (SAMe) and SAMe and APAP

treated (SAMe + APAP). Values represent Mean ± SEM, n=7-10 mice/group. Total hepatic glutathione levels were measured 4 h after injection (ip) of 300 mg/kg APAP. Unlike superscript letters indicate groups with statistical difference ($p<0.05$).

Figure 4. Total Hepatic Glutathione (GSH) Levels following NAC and APAP injection in C57BL/6 mice. Animals were randomly divided into vehicle (VEH), acetaminophen treated (APAP), NAC (1.25 mmol/kg) treated (NAC) and combined NAC and APAP treated (NAC+ APAP). Values represent Mean ± SEM, n=7-10 mice/group. Total hepatic glutathione levels were measured 4 h after injection (ip) of 300 mg/kg APAP. Groups with unlike superscripts are statistically different ($p<0.05$) from each other.

Figure 5. Comparison of equimolar doses of SAMe and NAC on Total Hepatic Glutathione (GSH) Levels following APAP injection Total hepatic glutathione levels were measured 4 h after ip injection of 300 mg/kg APAP. Groups represent: vehicle (VEH), acetaminophen treated (APAP), combined NAC (1.25 mmol/kg) and APAP treated (NAC+APAP) and combined SAMe (1.25 mmol/kg) and APAP treated (SAMe+APAP). Values represent Mean ± SEM, n=7-10 mice/group. Total hepatic glutathione levels were measured 4 h after injection (ip) of 300 mg/kg APAP. Groups with unlike superscripts are statistically different ($p<0.05$) from each other.

Figure 6. Total Hepatic Glutathione (GSH) Levels following high dose NAC and APAP injection in C57BL/6 mice. Animals were randomly divided into vehicle (VEH), acetaminophen treated (APAP), NAC (7.35 mmol/kg) treated (NAC) and combined NAC and APAP treated (NAC+ APAP). Values represent Mean ± SEM, n=4-5 mice/group. Total hepatic

glutathione levels were measured 4 h after injection (ip) of 300 mg/kg APAP. Groups with unlike superscripts are statistically different ($p<0.05$) from each other.

Figure 7. The Effect of SAMe on Glutathione disulfide (GSSG) Levels following APAP injection in C57BL/6 mice. Animals were randomly divided into vehicle (VEH), acetaminophen treated (APAP), SAMe (1.25 mmol/kg) treated (SAMe) and SAMe and APAP treated (SAMe + APAP). GSSG was expressed as percent of total GSH. Values represent Mean ± SEM, n=7-10 mice/group. Total hepatic glutathione levels were measured 4 h after injection (ip) of 300 mg/kg APAP. Unlike superscript letters indicate groups with statistical difference ($p<0.05$).

Figure 8. The Effect of NAC on Glutathione disulfide (GSSG) Levels following NAC and APAP injection in C57BL/6 mice. Animals were randomly divided into vehicle (VEH), acetaminophen treated (APAP), NAC (1.25 mmol/kg) treated (NAC) and combined NAC and APAP treated (NAC+ APAP). GSSG was expressed as percent of total GSH. Values represent Mean ± SEM, n=7-10 mice/group. Total hepatic glutathione levels were measured 4 h after injection (ip) of 300 mg/kg APAP. Groups with unlike superscripts are statistically different ($p<0.05$) from each other.

Figure 9. The Effect of high dose NAC on Glutathione disulfide (GSSG) Levels following high dose NAC and APAP injection in C57BL/6 mice. Animals were randomly divided into vehicle (VEH), acetaminophen treated (APAP), NAC (7.35 mmol/kg) treated (NAC) and combined NAC and APAP treated (NAC+ APAP). GSSG was expressed as percent of total

GSH. Values represent Mean ± SEM, n=4-5 mice/group. Total hepatic glutathione levels were measured 4 h after injection (ip) of 300 mg/kg APAP. Groups with unlike superscripts are statistically different ($p<0.05$) from each other.

Figure 10. Protein carbonyls in hepatic tissue treated with SAMe and APAP. Hepatic tissues were collected from mice 4 h after injection (ip) with VEH (lane 1), 1.25 mmol/kg SAME (lane 2), 300 mg/kg APAP (lane 3) and a combination of SAMe and APAP (SAMe+APAP lane 4) as described in the methods. Western blots were performed using an OxyBlot Kit to detect protein carbonyls. Gels were loaded with the same volume and concentration of protein. Typical blots are shown; bands were detected via chemiluminescence.

Figure 11. Protein carbonyls in hepatic tissue treated with NAC and APAP. Hepatic tissues were collected from mice 4 h after injection (ip) with VEH (lane 1), 1.25 mmol/kg NAC (lane 2), 300 mg/kg APAP (lane 3) and a combination of NAC and APAP (NAC+APAP lane 4) as described in the methods. Hepatic tissues were collected and processed as described in the methods. Western blots were performed using an OxyBlot Kit to detect protein carbonyls. Typical blots are shown; gels were loaded with the same amount of protein in identical volumes.

Figure 12. 4-HNE adducted proteins 4h after treatment with SAMe and APAP. Hepatic tissues were collected from mice 4 h after injection (ip) with VEH (lane 1), 1.25 mmol/kg SAME (lane 2), 300 mg/kg APAP (lane 3) and a combination of SAMe and APAP (SAMe+APAP lane 4) as described in the methods. Western blots were performed using an anti-4HNE primary antibody with a peroxidase conjugated secondary antibody. Gels were loaded with the same

volume and concentration of protein based on mass. Bands were detected via chemiluminescence.

Figure 13. 4-HNE adducted proteins 4 h after treatment with NAC and APAP. Hepatic tissues were collected from mice 4 h after injection (ip) of APAP or VEH. Typical blots are shown for 2 animals from the: VEH (lanes 1-2), 1.25 mmol/kg NAC (lane 3-4), 300 mg/kg APAP (lane 5-6) and a combination of NAC and APAP (NAC+APAP lanes 7-8) as described in the methods. Hepatic tissues were collected and processed as described in the methods. Western blots were performed using an anti-4HNE primary antibody with a peroxidase conjugated secondary antibody. Gels were loaded with the same volume and concentration of protein based on mass. Bands were detected via chemiluminescence.

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TABLE 1. Effect of NAC and SAMe on body weight and liver weight. All values represent Mean ± SEM with n= 4-10 mice/group. Groups were treated with: vehicle (VEH, water), SAMe (500 mg/kg, 1.25 mmol/kg), acetaminophen APAP (300 mg/kg), n-acetylcysteine (NAC 204 mg/kg, 1.25 mmol/kg) and high dose n-acetylcysteine (NAC-H 1200 mg/kg, 7.35 mmol/kg) or a combination of SAMe+APAP, NAC+APAP and NAC-H+APAP.

Group	Body weight (g)	Liver Weight (g/100 g body wt)
VEH	18.00±0.30	0.51±0.01[a]
SAMe	18.00±0.30	0.51±0.01[a]
APAP	17.80±0.36	0.59±0.01[b]
SAMe+APAP	17.80±0.36	0.52±0.02[a]
VEH	17.60±0.40	0.46±0.02[a]
NAC	18.00±0.60	0.48±0.01[a]
APAP	18.40±0.83	0.58±0.01[b]
NAC+APAP	18.20±0.36	0.58±0.01[b]
VEH	19.60±0.40	0.46±0.01[a]
NAC-H	17.60±0.40	0.50±0.01[b]
APAP	18.40±0.75	0.56±0.01[c]
NAC-H+APAP	19.50±0.50	0.50±0.02[b]

[abc]Superscript letters indicate groups with statistical difference ($p<0.05$). No differences were observed in the body weights between groups.

TABLE 2. Effect of NAC and SAMe on ALT levels. All values represent Mean ± SEM with n= 4-10 mice/group. Groups were treated with: vehicle (VEH, water), SAMe (500 mg/kg, 1.25 mmol/kg), acetaminophen APAP (300 mg/kg), n-acetylcysteine (NAC 204 mg/kg, 1.25 mmol/kg) and high dose n-acetylcysteine (NAC-H 1200 mg/kg, 7.35 mmol/kg) or a combination of SAMe+APAP, NAC+APAP and NAC-H+APAP.

Group	ALT (U/L)
VEH	80.95±18.69[a]
SAMe	373.65±70.12[a]
APAP	8533.33±623.10[b]
SAMe+APAP	2811.51±346.91[c]
VEH	83.97±18.94[a]
NAC	218.73±60.58[a]
APAP	9107.58±873.13[b]
NAC+APAP	10208.11±1252.37[b]
VEH	34.29±13.08[a]
NAC-H	107.94±51.91[a]
APAP	6673.02±1376.64[b]
NAC-H+APAP	606.35±215.51[a]

[abc]Superscript letters indicate groups with statistical difference ($p<0.05$). No differences were observed in the body weights between groups.

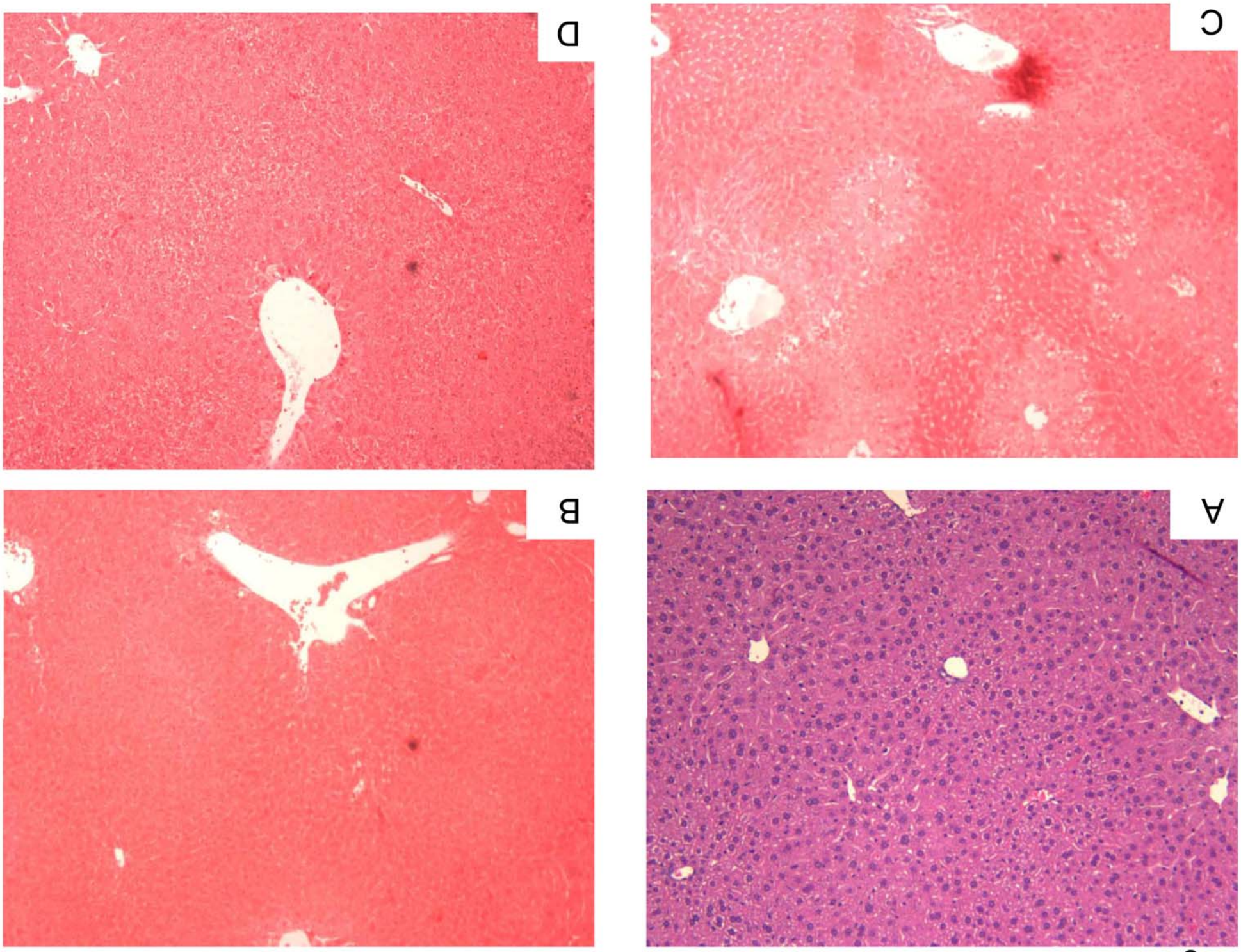

Fig. 1

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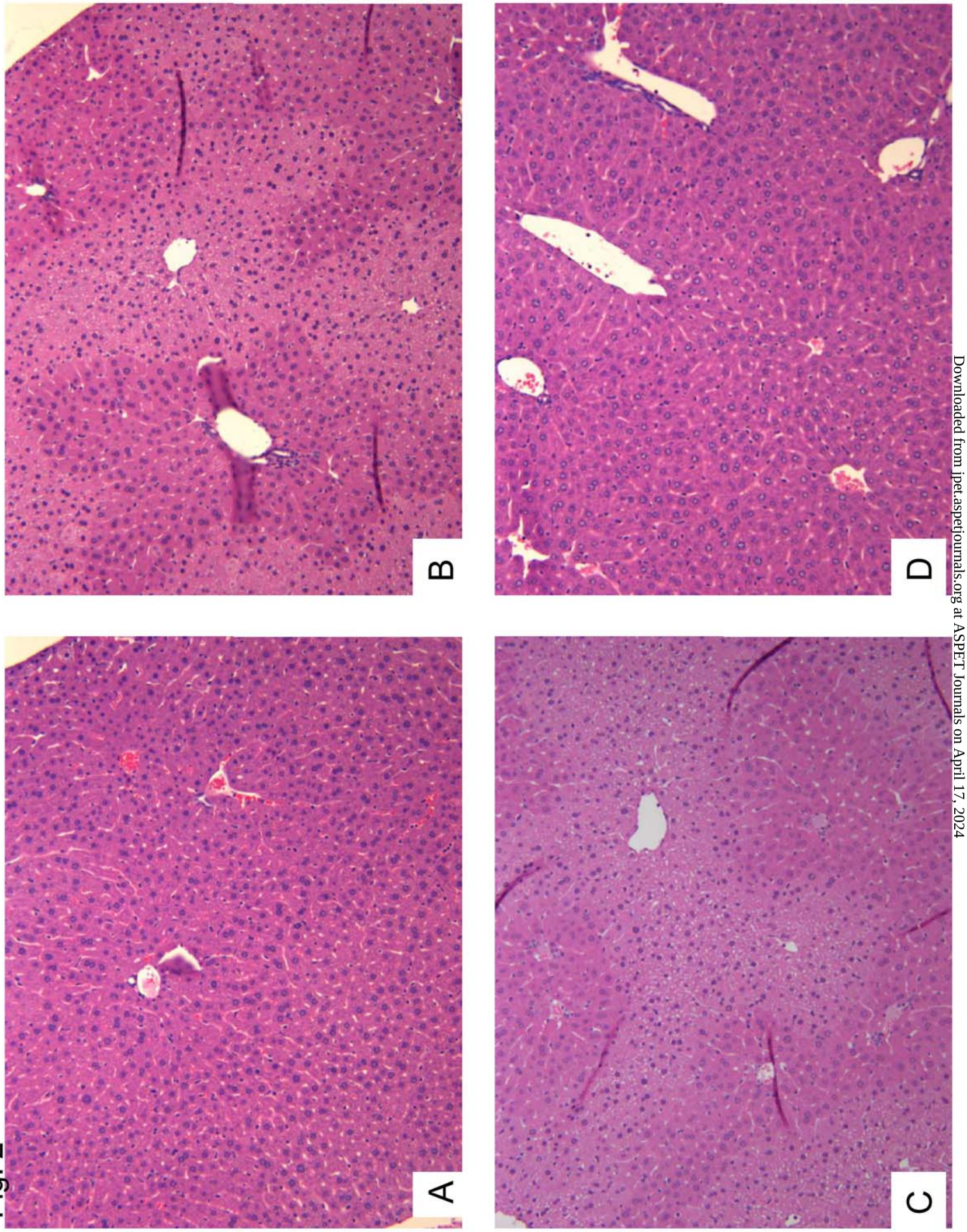
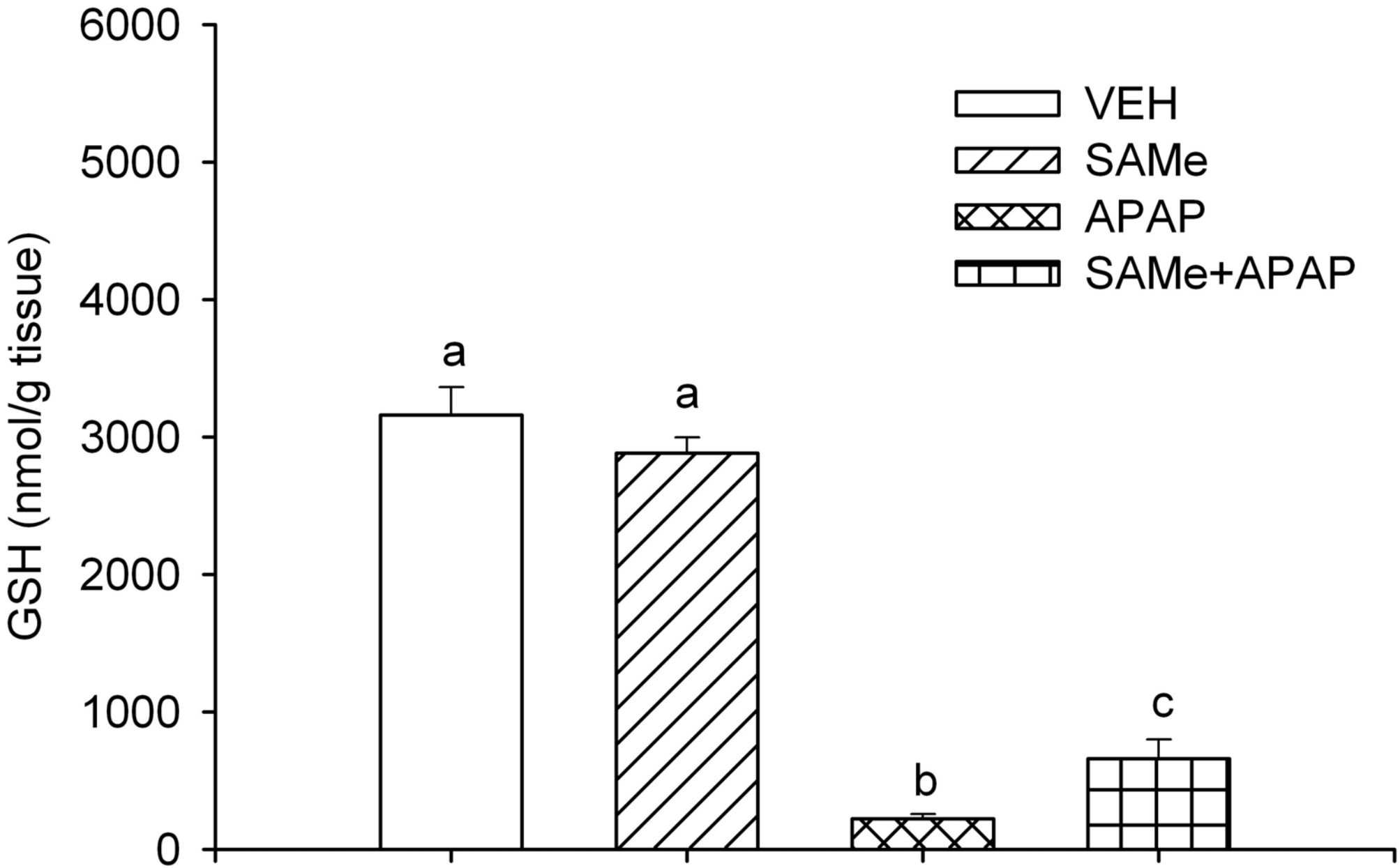


Fig. 2

Fig. 3



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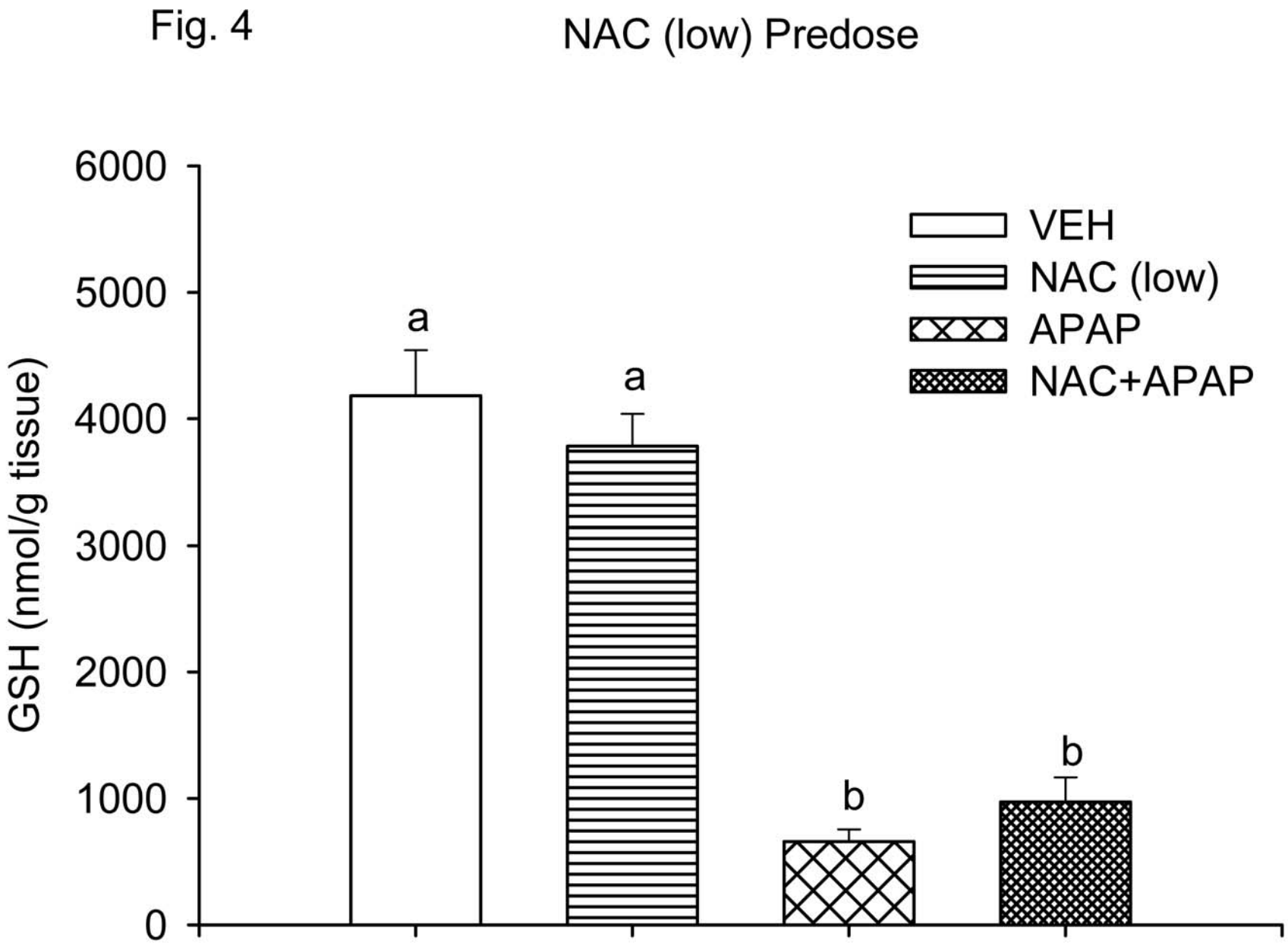

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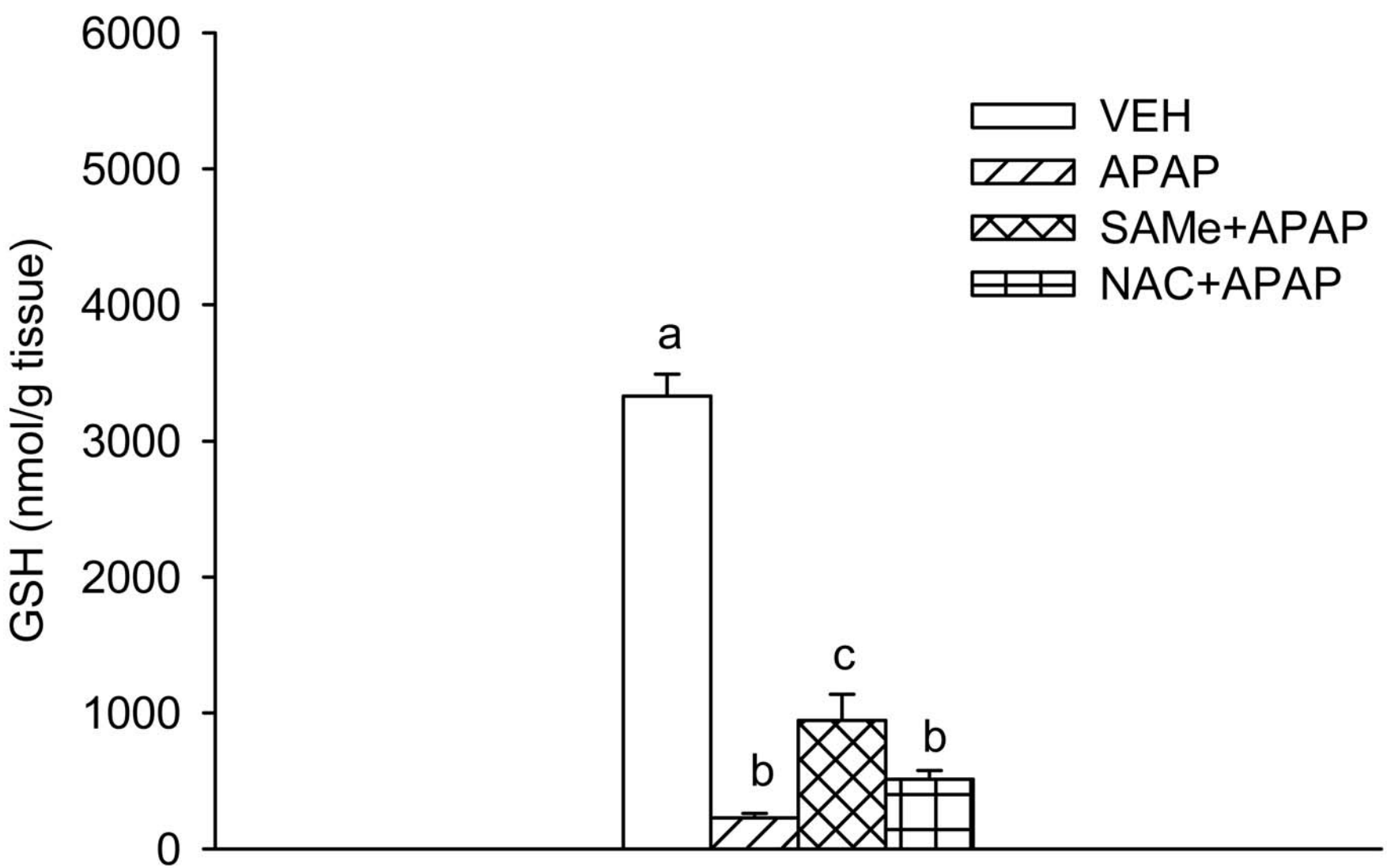

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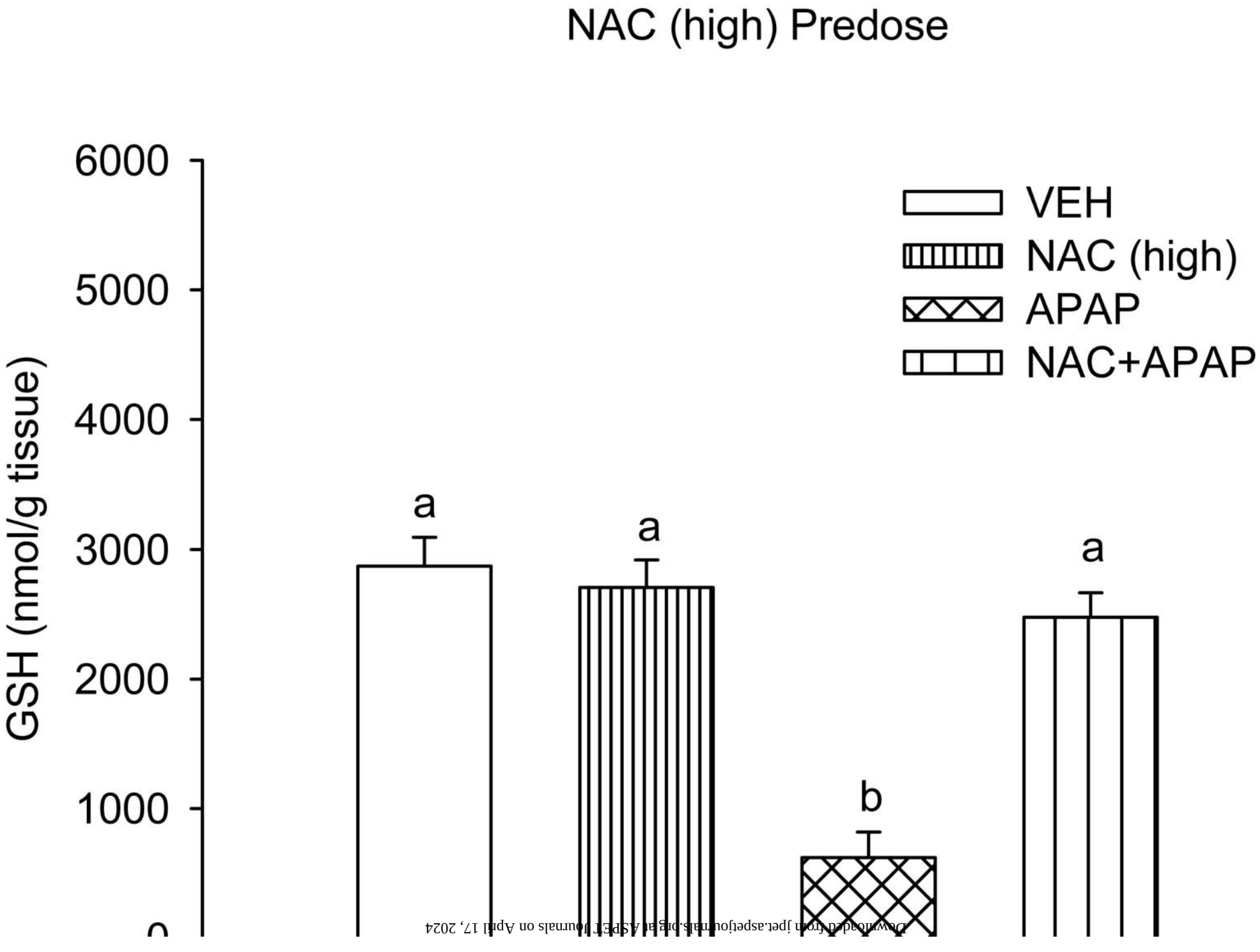

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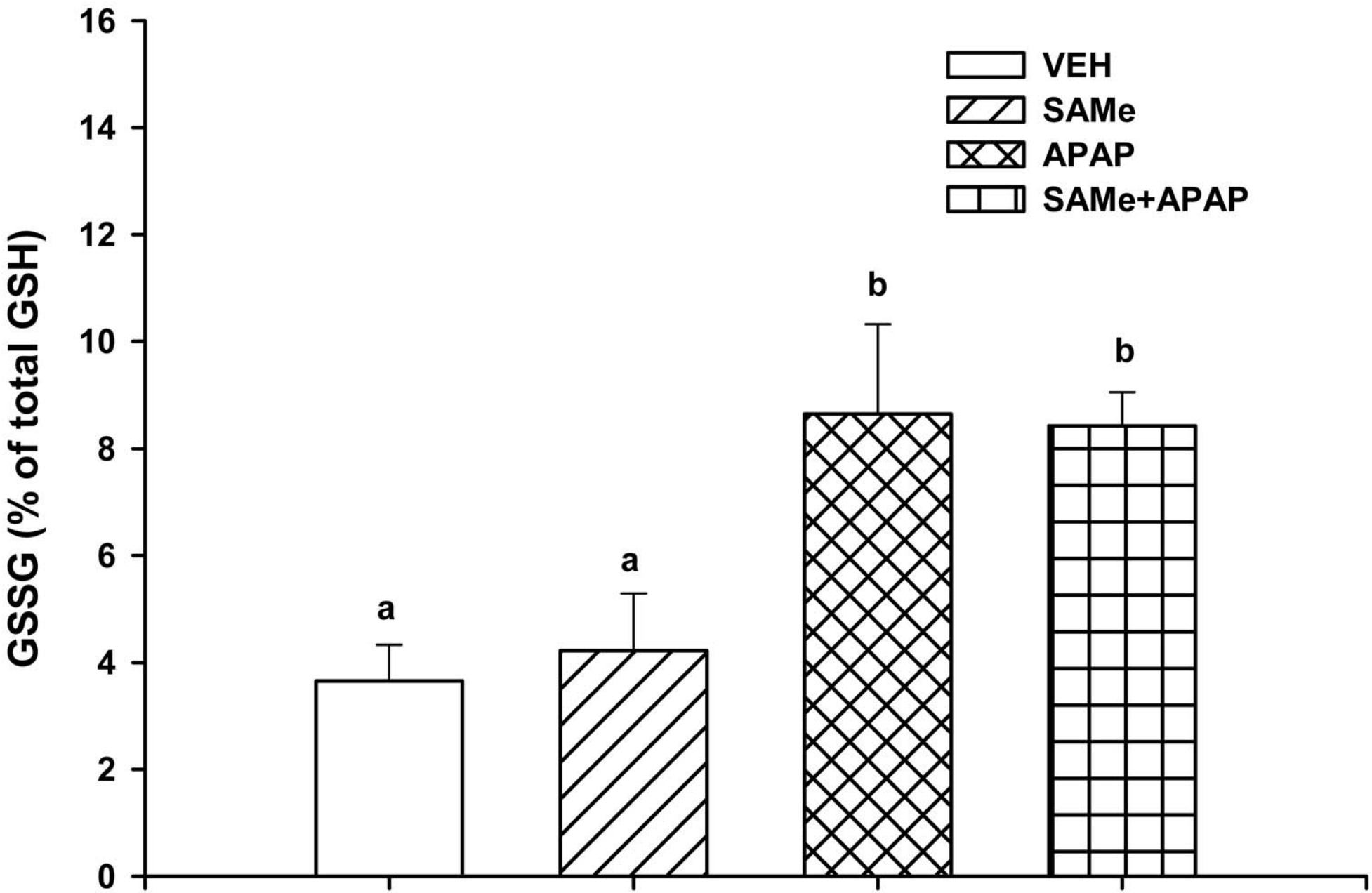

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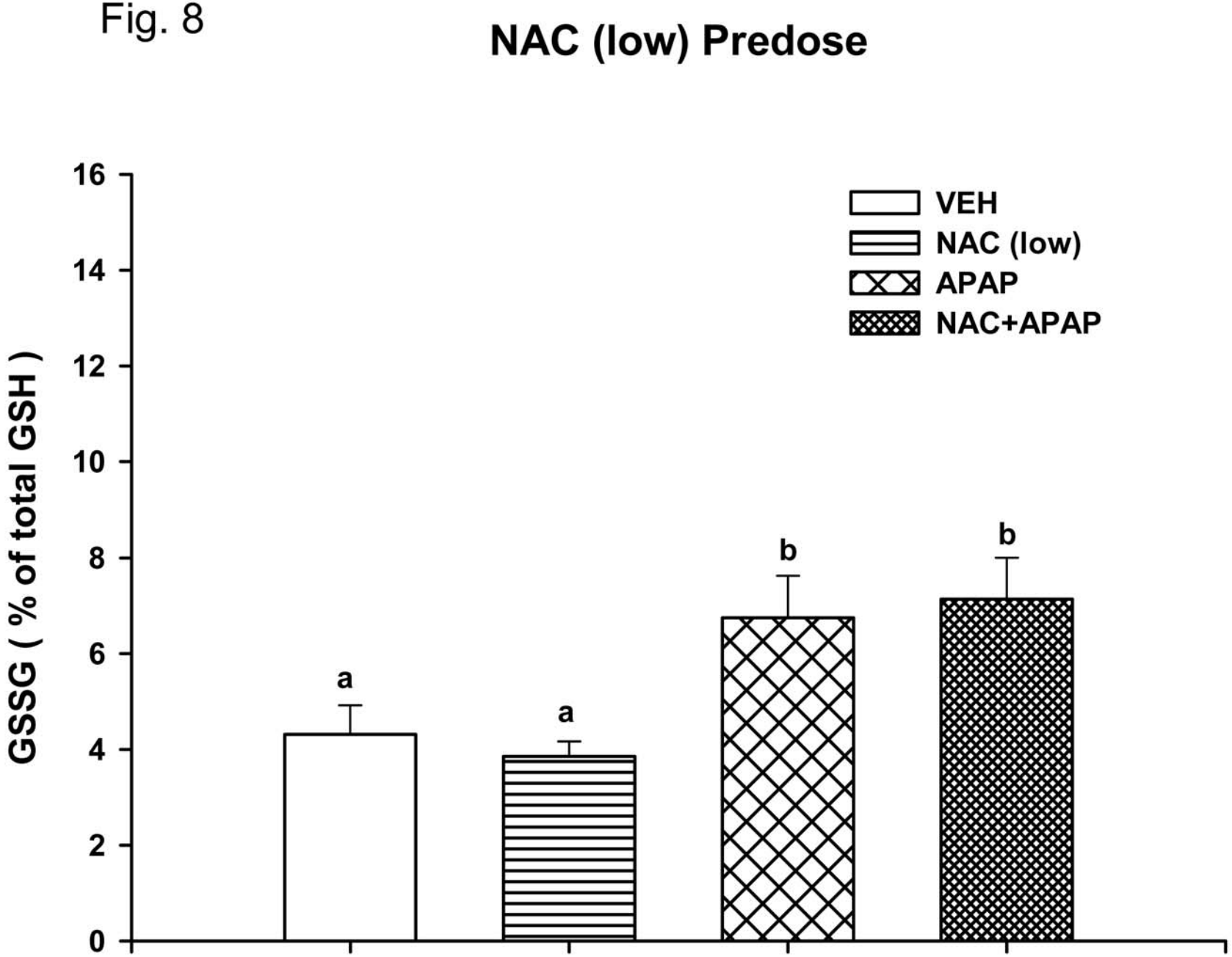

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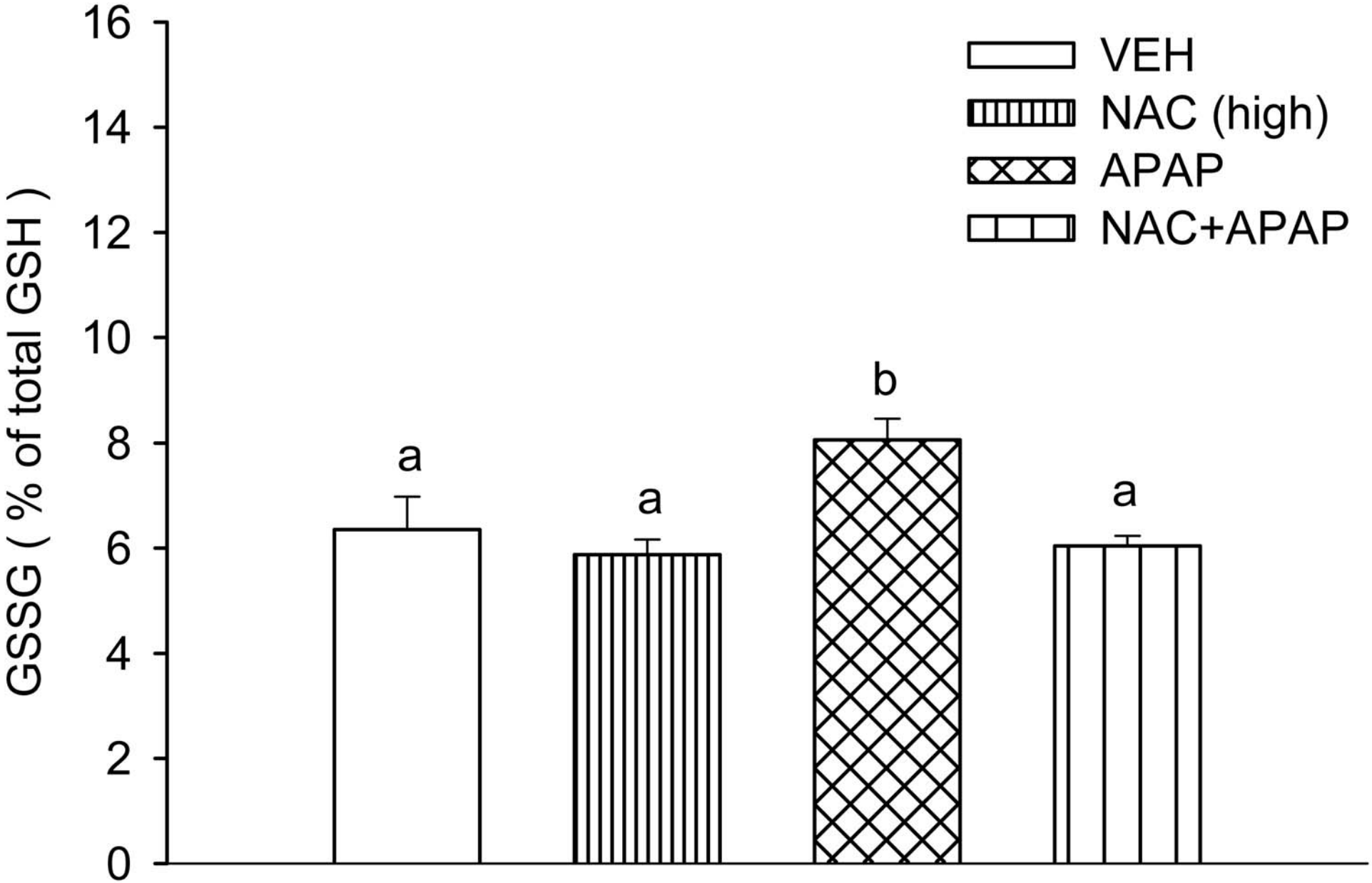

Fig. 10

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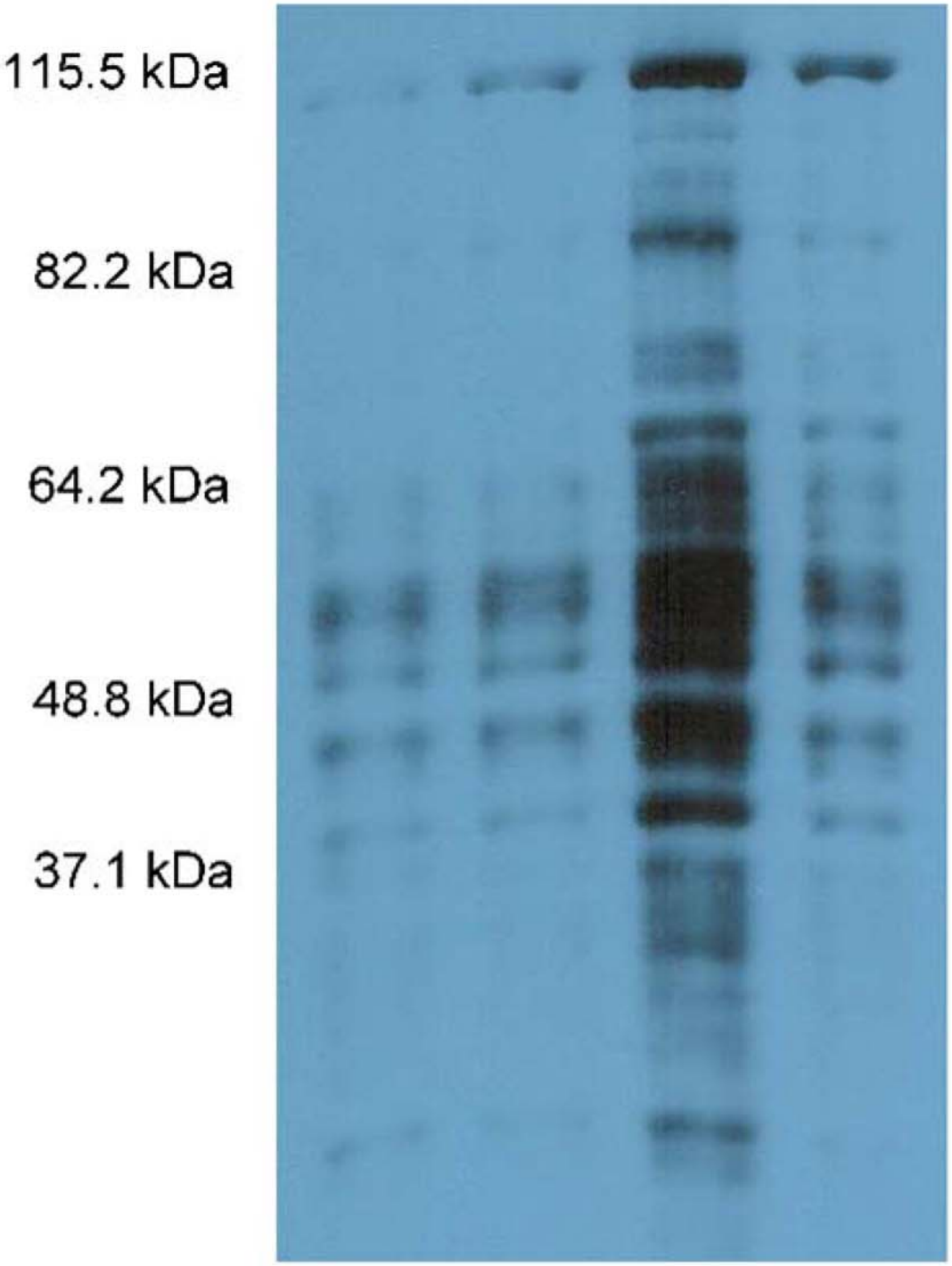

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Fig.11

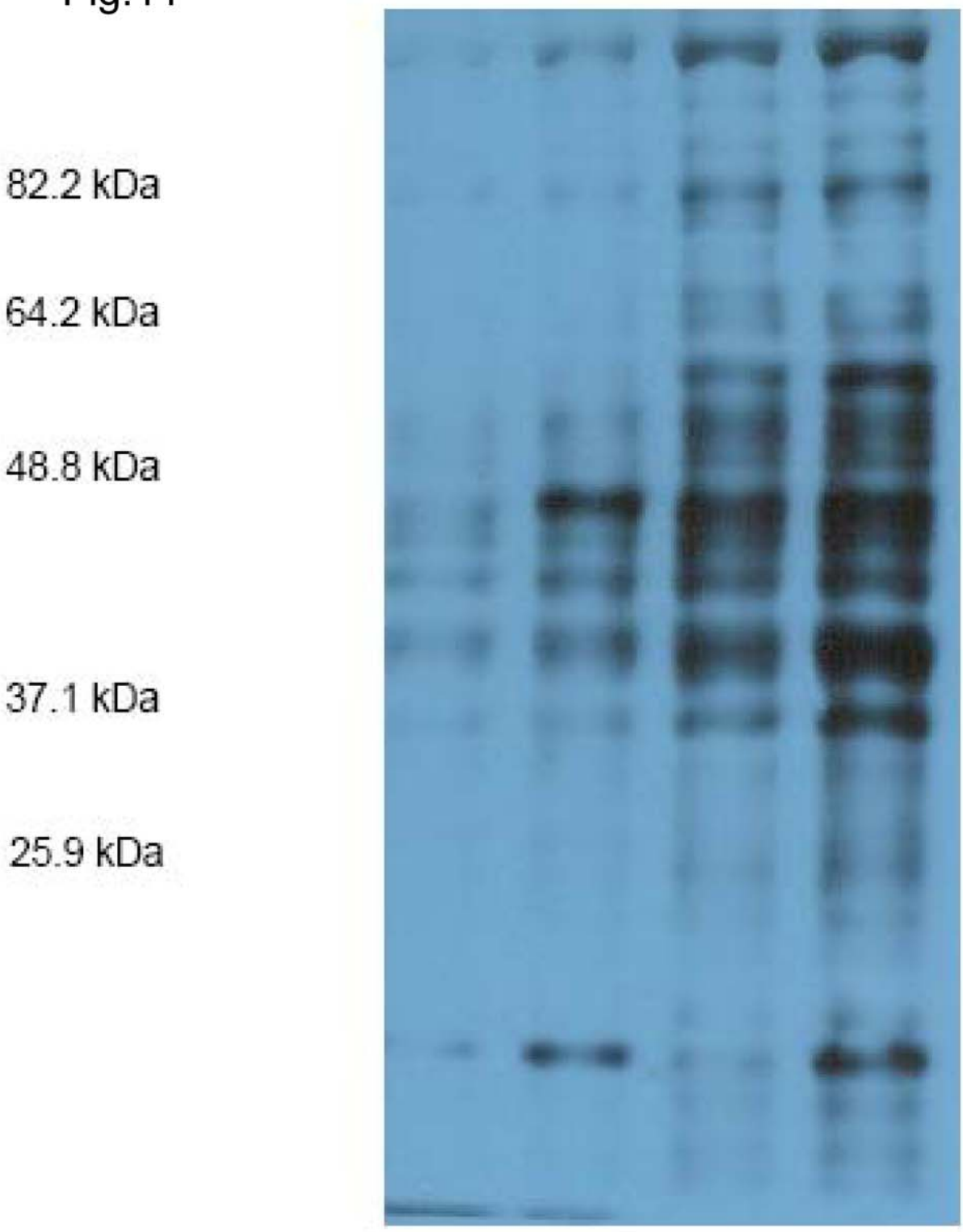

Fig. 12

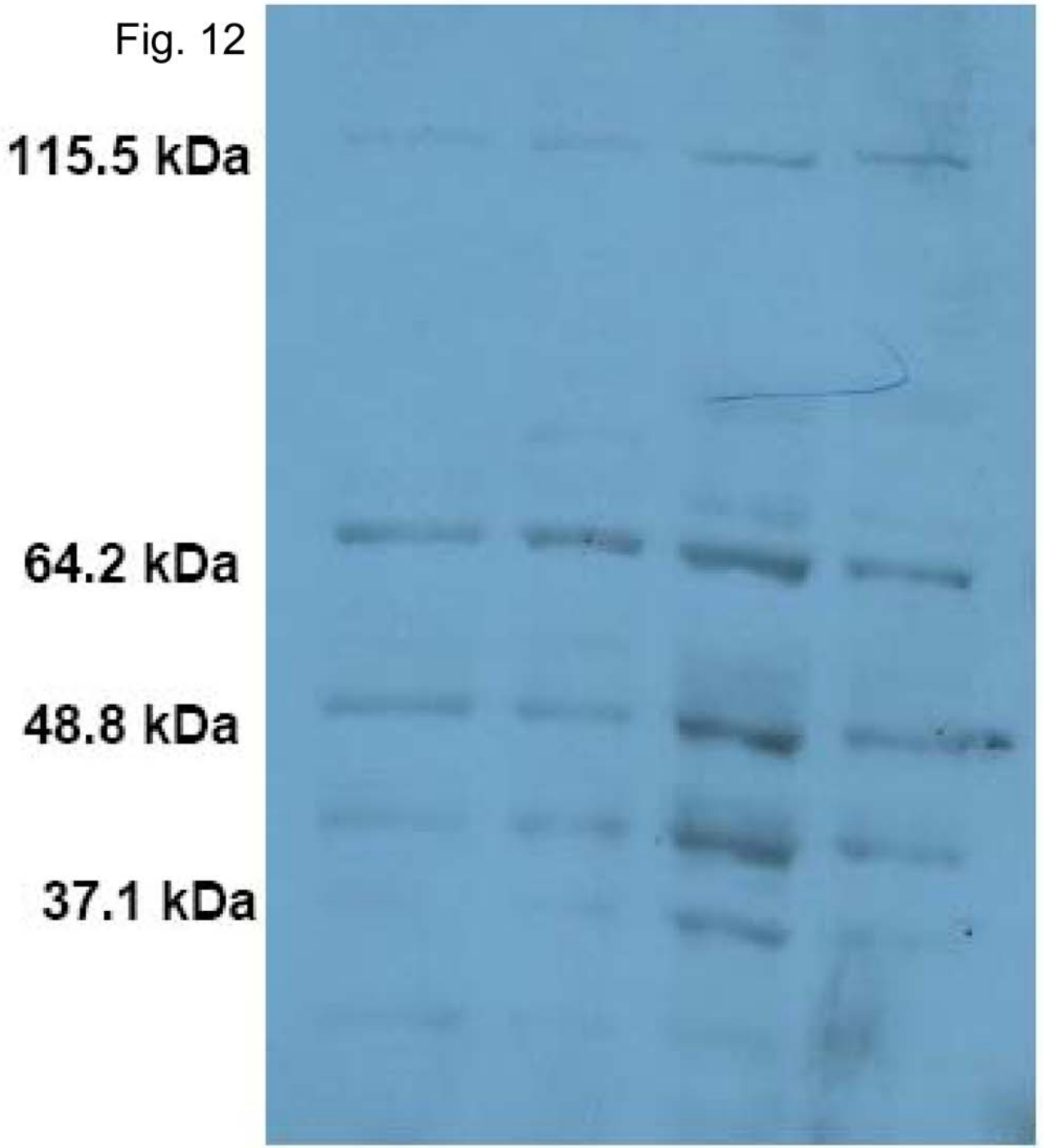

Fig. 13