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Title page

Gender differences in the bile acid profiles of APP/PS1 transgenic AD mice

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Abstract

Alzheimer's disease (AD) is a neurodegenerative disease and presents in the accumulation of amyloid and neurofibrillary tangle. The association between modulation of gut symbiotic microbes with neurological disease *via* bidirectional gut-brain axis has been well documented. Bile acid (BA) pools in the enterohepatic circulation could be valuable for probing complex biochemical interactions between host and their symbiotic microbiota. Herein we investigated the levels of 28 BAs in several compartments in enterohepatic circulation (including jejunal, ileum, cecum, colon and feces, plasma and liver tissue) by employing an APP/PS1 induced transgenic AD mouse model. We found that BA profiles in AD mice were gender specific. We observed decreased levels of taurine-conjugated primary BAs (TUDCA, TCA, T-α-MCA and T-β-MCA) and increased levels of secondary BA (iso-DCA) in plasma and liver extracts for female AD transgenic mice. In contrast, increased levels of TDCA in liver extracts and decreased levels of T-β-MCA in jejunal content were noted in male AD mice. These observations suggested that perturbations of BA profiles in AD mice displayed clear gender variations. Our study highlighted the roles of gut microbiota on neurodegenerative disease, which could be gender specific.

Keywords: Bile acid, Gender difference, Alzheimer's disease, Metabonomics, enterohepatic circulation.

Introduction

Alzheimer's disease (AD) is a progressive cognitive disorder and has been characterized by accumulation of extracellular beta amyloid plaques and intraneuronal neurofibrillary tangle. AD affects nearly 50 million people aged over 65 years old and causes social-economic burden of more than 600 billion dollars per year worldwide. AD has become one of the top causes of disabilities in later life. Since the etiology of AD is still not fully understood (Querfurth and LaFerla 2010), there is no effective cure or strategy to slow down disease progression. Furthermore, both epidemiological studies and rodent models demonstrate a gender-specific pattern in the incidence of AD with higher risk in women. For example, life time risk for developing AD is two-fold higher in women than in men (Seshadri et al., 1997, Yang et al., 2017). Severe amyloid plaque is reported to accumulate in female transgenic mice model (Wu et al., 2016). Such susceptibility might be due to female-specific risk gene for AD development (Li et al., 2017). Therefore, exploring the underlying gender-specific mechanisms is essential for AD management in the future.

Recently, studies have revealed that human gut symbiotic microbes could be an intermediator to modulate neurological diseases *via* a bidirectional gut-brain axis (Lynch and Pedersen, 2016, Sarkar et al., 2016). Germ-free mice display increased motor activity and reduced anxiety-like behavior (Diaz Heijtz et al., 2011). Studies have suggested that some social abnormality could be potentially prevented by colonization with species of *Clostridium buryicum* (Xie et al., 2015), *lactobacillus rhamnosus* (Bravo et al., 2011) or *bacteroides fragilis* (Hsiao et al., 2013). In addition, decreased abundances of anti-inflammatory butyrate producing bacteria, such as, *Butyrivibrio fibrisolvens*, *Coprococcus* and *Roseburia* in feces, are found to be closely linked with Parkinson's diseases (Keshavarzian et al., 2015) and amyotrophic lateral sclerosis (Wu et al., 2015). Harach et al also found a significant

perturbation of gut microbiota, such as, decreased abundance of *Akkermansia* and *Allobaculum*, increased abundance of *Rikenellaceae*, in feces of AD transgenic mice, which showed the direct connection between microbiota and AD in mouse model (Harach et al., 2017). Such connection is verified by the increased cerebral Aβ amyloid plaques in the recolonization of germ-free APP transgenic AD mice with microbiota from conventionally-raised APP transgenic mice (Harach et al., 2017). Gut immune function associated with perturbation of microbial taxa in transgenic AD mice are suggested to be the underlying reason for the observed cerebral Aβ amyloid accumulation in the re-conventionalized AD mice (Harach et al., 2017). In consistence with the immune responses to perturbations of gut microbes, Geng et al also highlighted that gut microbiota is a driving factor in promoting Th1 cells infiltration, leading to M1 microglia-predominated neuro-inflammation in AD progression (Wang et al., 2019). A study also suggests that modulating gut microbiome through nutritional interventions can be an effective strategy to prevent the development of AD (Pistollato et al., 2016). These evidences together indicate the important roles of microbiota in the development of neurodegenerative diseases (Zhan et al., 2016, Harach et al., 2017).

Bile acids (BAs) are the end products of cholesterol catabolism and can facilitate absorption of lipids in the intestine and regulate cholesterol homeostasis in the liver (Lefebvre et al., 2009, de Aguiar Vallim et al., 2013). The metabolic perturbation of BA biosynthesis will ultimately result in the alterations of cholesterol metabolism, which has been found in AD (Puglielli et al., 2003). The gut microbiota are known to play important role in modulating BA homeostasis reciprocally (Wahlstrom et al., 2016). For example, the gut microbiota could modulate signaling regulation of BAs *via* nuclear farnesoid X receptor (FXR) and G protein-coupled membrane receptor 5 (TGR5), and *vice versa*. The BAs also can modulate gut microbial

compositions through innate immune genes in small intestine (Wahlstrom et al., 2016). Therefore, the integrated metabolism of BA pools could be valuable for characterizing complex biochemical interactions between host and their symbiotic microbiota under physiological and pathophysiological status (Martin et al., 2007, Joyce and Gahan, 2016). Previous metabolomic study using ultra performance of liquid chromatography coupled with mass spectrometry (UPLC-MS) reveals up-regulation of glycocholate (GCA), glycodeoxycholate (GDCA) and glycochenodeoxycholate (GCDCA) in plasma profiles for mild cognitive impairment (MCI) and AD patients (Marksteiner et al., 2018). However, recently, Pan et al suggests that decreased levels of cholic acid (CA) and taurocholic acid (TCA) in plasma and brain specimen, respectively, of AD patients (Pan et al., 2017). These discrepancies not only addressed the important roles of BAs in AD, but also suggested the unmet needs for clarifying the issue further.

The objective of this work is to assess the topographical BA variations associated with AD in both genders by employing an APP/PS1 double mutations mouse model. Herein, we comprehensively analyzed the fluxes of BAs in plasma, liver extracts, four intestinal contents (jejunal, ileal, cecal and colonic contents) and feces of AD mice using targeted BAs quantification with UPLC-MS method. We discovered gender specific BA profiles in AD mice. Our results provided further evidence for involvement of gut microbiota through modification of BA metabolism in neurodegenerative diseases.

Material and Methods

Experimental Procedures

The animal experiment procedure and sample collection were described previously (Wu et al., 2016). Briefly, a total of 37 double mutant transgenic mice (APPswePSEN1dE9) aged 4 weeks

old were purchased from the national animal resource center at Nanjing University (Nanjing, China) (Francis et al., 2009). All mice were housed in standard SPF animal laboratory facility at Wuhan University. The animal procedures were approved by the ethics committee for animal care at Wuhan University (No 398-2006). The mice were divided into four groups: female transgenic group (FT, n=9) and their female wild-type counterparts (FW, n=10), male transgenic group (MT, n=9) and their male wild-type counterparts (MW, n=9).

At the age of 9 months, fecal samples were collected one day before sacrifice and fasting blood samples were collected from orbital venous plexus of mice into sodium heparin containing Eppendorf on the day of sacrifice. The mice were sacrificed by cervical dislocation under isoflurane anesthesia. The middle lobe of liver from each mouse was excised and the intestinal contents from jejunum, ileum, cecum and colon were also collected. All the collected samples were snap-frozen in liquid nitrogen and stored at -80°C till BA measurements.

Chemicals and Reagents

28 BA standards including 15 unconjugated BAs and 13 conjugated BAs (7 taurine-conjugated and 6 glycine-conjugated BAs) were purchased from Steraloids Inc. company (Newport, RI, USA). Their nomenclatures, abbreviations and categories based on their origins (12 primary BAs and 16 secondary BAs) were listed in Table 1 (Claus et al., 2008, Wahlstrom et al., 2016). The deuterated BAs, [2,2,4,4-D_4]-DCA (DCA-d_4), [2,2,4,4-D_4]-CA (CA-d_4), [2,2,4,4-D_4]-CDCA (CDCA-d_4), [2,2,4,4-D_4]-LCA (LCA-d_4), [2,2,4,4-D_4]-GCDCA (GCDCA-d_4) and [2,2,4,4-D_4]-GCA (GCA-d_4) obtained from Steraloid Inc. were served as internal standards. These internal standards covered all bile acids structural unit. The HPLC-grade formic acid, acetonitrile, and methanol were obtained from Sigma-Aldrich (St. Louis, USA). The deionizing and deoxidizing

water was acquired from a Millpore Elix Advantage purification system (Merck Millipore, Germany).

Table 1. The nomenclatures and abbreviations for the measured bile acids with categorization information.

UPLC-MS/MS based BA analysis

The BAs were quantified with a previously optimized method (Lin et al., 2019). Briefly, 5 milligram (mg) liver samples were extracted with 1 mL of precooled methanol-water mixture (2:1, v/v, containing 0.005% HCOOH and 50 uL of internal standard mixture). The mixed solution was homogenized with a tissuelyser (QIAGEN, Hilden, Germany) at 20Hz for 90s for three times. Following centrifugation for 10 min, the supernatant was filtered through 0.22um membrane and transferred into chromatographic vial for further MS measurement. In parallel, all intestinal contents and feces samples were divided into two parts of 5 mg: one part was extracted the same way as liver tissues for BA analysis and the other part was used for the measurement of water content. 30uL plasma were homogenized with 1mL precooled methanol-water mixture and centrifuged directly to obtain supernatant for BA analysis.

Liquid chromatographic separation was achieved using a Kinetex® Core-Shell 2.6 µm C18 column (100×2.1mm, 2.6µm, Phenomenex, USA) maintained at 45^{o}C, with an injection volume of 1 uL. MilliQ water containing 0.005% HCOOH (A) and acetonitrile containing 0.005% HCOOH (B) were used as mobile phase following the gradient elution: 0-2 min 23% B, 2-6 min 33% B, 6-11 min 34% B, 11-15 min 70% B at a flow rate of 0.6 ml/min. The N_2 was used as a drying gas at 10 L/min and source temperatures was 350^{o}C. The BA measurements were performed on Agilent 1290 HPLC-MS/MS (Agilent Technologies, USA) equipped with 6460 triple quadrupole MS and an electrospray ionization source operating in negative detection mode

(ESI-). Mass spectrometry was performed on a multiple reaction monitoring (MRM) system. Each BA was identified by parent ion and daughter ion selected from each standard during method development and aided with their retention times. The quality control sample was acquired for every 8 samples to monitor the quality of measurement. The quantification was performed against area of structurally similar internal standards using Mass Hunter (Agilent Technologies, USA). The results were expressed as nmol per liter of plasma or per gram of liver or dry weight of intestinal content.

Statistical analysis

All statistical analysis was performed using the statistical package SPSS software (version 20 Chicago, IL, USA) and Graphpad Prism software (version 7, Graphpad software Inc., San Diego, CA, USA). D'Agostino and Pearson omnibus normality was used to check the normal distribution and homoscedasticity for each dataset. The one-way ANOVA with FDR method of Benjamini and Hochberg (B-H) correction was used for comparison between transgenic mice and its wide-type littermates for normal distribution dataset, otherwise non-parametric Kruskal-Wallis test with FDR method of B-H correction was used. Results were expressed as Mean ± SEM. The figures were plotted in the Graphpad 7.0 environment. Results were considered as significant when the adjusted p value is less than 0.05.

Results

BA profiles in different compartments

A panel of BAs in four intestinal contents (jejunal, ileal, cecal and colonic content), feces, liver extracts and plasma from a female transgenic mouse and its wild type counterpart were detected with the reference to standards (Figure 1). The BA concentrations and their

proportions in different matrices in different genders were listed in table 2 and 3. Since the high variability of all BAs in various sample matrices, the BAs concentration were expressed as log-transformed values. Visual inspection of the data revealed bio-matrix specific BA profiles. For example, the hepatic and plasma BA profiles were dominated by taurine conjugates, with TCA presented at the highest levels. The most concentrated bile acid was unconjugated BAs, such as CA, and presented mainly in plasma and liver extracts. The intestinal BA compositions were different from those of plasma and liver extracts. Compared to large intestinal (cecum and colon) content and fecal extracts, the small intestinal (jejunum and ileum) content contained higher levels of glycine conjugated BAs and with more diversity. Along the intestinal compartment, levels of taurine-conjugated and glycine-conjugated BAs decreased and levels of secondary BAs (such as LCA, DCA and 12-kDCA) increased from proximal to distal intestine.

Figure 1. Relative bile acid concentrations from seven compartments in enterohepatic circulation of female transgenic mice and their wild type littermates.

Table 2. Concentrations of bile acids in different biological matrices of female APP/PS1 transgenic mice and their wildtype littermate(Hofmann and Marschall, 2018).

Table 3. Concentrations of bile acids in different biological matrices of male APP/PS1 transgenic mice and their wildtype littermate.

AD associated BAs variations in intestinal contents and feces

We further compared bile acids compositions between the APP/PS1 double mutations mice and their counterparts for each biological matrix for male and female separately. Significant variations in the levels of BAs were observed in jejunal content between transgenic AD mice and their corresponding wide type counterparts for both male and female mice (Figure 2).

No significant change in the levels of BAs in other regions of intestinal tract or feces was observed between transgenic mice and wild type mice in both genders.

Figure 2. Concentrations of BAs in jejunal contents between transgenic mice and their comparable wild type counterparts.

The jejunal BA profiles in transgenic female mice showed elevated levels of glycine-conjugated BAs, which were mainly attributed by the increases in the levels of primary bile acids, GUDCA and GDCA ($p<0.05$). The other increased levels of jejunal BAs in female transgenic mice included primary BA, such as, α-MCA and β-MCA, and a secondary BA, i.e. ω-MCA. On the contrary, there was a significant decrease in the levels of jejunal T-β-MCA in male transgenic mice when compared to their wildtype controls, which resulted in the decreases in the levels of total BAs in the male transgenic mice (Figure 2).

AD associated BAs variations in plasma and liver extracts

TCA was the dominating BA in plasma and liver extracts. Both plasma and liver extracts of female transgenic mice showed significantly decreased levels of taurine conjugated compared to their wild type counterparts, such as, TCA, T-α-MCA, T-β-MCA and TUDCA (Figure 3A and 3C). In liver extracts, the levels of secondary BA, iso-DCA, was increased for female transgenic AD mice (Figure 3A). However, in male transgenic mice, only increased level of TDCA was observed in the liver extracts compared with their wild type littermates (Figure 3B). No significant difference was found for unconjugated and glycine-conjugated BAs in plasma and liver extracts for both genders.

Figure 3. Concentrations of BAs between transgenic AD mice and their comparable wild type counterparts in liver extracts (A, B) and plasma samples(C, D).

Discussion

Human gut symbiotic microbes have been increasingly recognized to play important roles in modulating neurological diseases *via* a bidirectional gut-brain axis (Foster and Neufeld, 2013). BAs are one category of the mediates reflecting the consequences of the functional crosstalk between hosts and gut microbiota. Hence, in the current investigation, we quantified compartmental BA profiles throughout the enterohepatic circulation in a transgenic APP/PS1 double mutant AD mouse model with an aim to explore the associations of BAs in AD. The results have illustrated that marked compartmental BA changes in different topographical intestinal contents, especially in jejunal content, plasma and liver extracts were associated with AD. These were mainly manifested in the decreases in the levels of taurine-conjugated primary BAs, and the increases in the levels of secondary BA iso-DCA. Most noticeably, such changes of BA profiles associated with AD were gender dependent.

Gender differences in bile acid profiles have been reported previously, including physiological state (Xie et al., 2015) and pathophysiological diseases, such as Parkinson's disease (Haaxma et al., 2007) and cardiovascular disease (Regitz-Zagrosek et al., 2006). Xiang et al found that levels of BA in fasting plasma are gender specific, however, no gender variation is found in the expression levels of CYP7A1, the rate-limiting enzyme of bile acid production in a Finnish population (Xiang et al., 2012). Gender variations are observed in a Swedish population (Gälman et al., 2011) and a healthy Chinese population (Xie et al., 2015), where the levels of circulating 7a-hydroxy-4 -cholestene-3-one (C4), a reliable intermediate in BA synthesis, are 30% higher in healthy men than in women. However, it is in contrast to the observation in mice, where BA synthesis and pool size are lower in male mice than in female mice (Turley et al., 1998, Fu et al., 2012). In our study, we also found higher levels of BA pool

size in female mice compared to male ones (data not shown), which is consistent with the distributions of BAs in mice (Dawson et al., 2003). Our previous investigation showed that AD plaque and metabolic perturbations are more severe in female transgenic AD mice than male mice (Wu et al., 2016), which demonstrated the gender differences in AD mouse model. Such differences addressed the necessity to scrutinize effects of AD on BA profiles with a view of a gender-specific association. Herein we investigated gender-specific BA profiles by employing an AD (APP/PS1) transgenic model, which has the benefit of eliminating the confounding factors of aging, nutrition and exogenous medical treatment. These factors will contribute to the variations in BA profiles and are otherwise difficult to control in elder population. We observed profound gender-specific BA metabolic alterations in jejunal contents, plasma and liver extracts of female AD mice compared to the control mice (Figure 2 and 3). Although Domínguez et al showed that the effect of gender on BA profiles is much less intense than the diseased state (González-Domínguez et al., 2015), in our study, gender specific BA profiles was noted in disease state.

Over all, decreased levels of taurine conjugated BAs were observed in transgenic AD female mice, which was mainly contributed by the decreases in the levels of primary BAs, such as TCA, TUDCA, T-α-MCA and T-β-MCA in plasma and liver extracts of female AD mice (Figure 3). Cholesterol is precursor of these taurine-conjugated BAs and also an important compound for maintaining physiological functions (Puglielli et al., 2003). The observed decreased levels of taurine conjugated BAs are consistent with the accumulated cholesterol found in plasma and liver extracts of female transgenic AD mice from our previous studies (Wu et al., 2016). The

Figure 4. Summarized alterations of bile acid metabolism induced by double APP/PS1 transgenic mutations in mice.

accumulation of cholesterol could trigger synaptic dysfunction and neuronal apoptosis in AD (Cutler et al., 2004). These accumulated cholesterol and decreased taurine-conjugated primary BAs implied the depressed conversion from cholesterol to CA and its downstream primary BAs in female AD mice (Figure 4) . Such inhibition is also supported by the expression levels of CYP7A1, an important enzyme for degradation of cholesterol to produce CDCA, which is significantly decreased by 50% in AD subjects (Yau et al., 2003). Our results are also consistent with the reduced levels of serum primary BAs and their glycine and taurine conjugated forms found in AD population (MahmoudianDehkordi et al., 2019). Moreover, the hydrolysis product of the taurine-conjugated BAs—taurine, an organic compound for membrane stabilization, is also decreased because of the above depressed conversion from cholesterol to taurine conjugated BAs in AD. Interestingly, Louzada and Menzie found that taurine treatment could prevent β-amyloid induced neurotoxicity through activation of GABA receptors, which confirmed the relevant role of taurine and its conjugates in AD (Louzada et al., 2004, Menzie et al., 2014). Taking these together, reduced cholesterol metabolism *via* bile acid production could play important role in AD pathogenesis, particularly in female.

TUCDA is another primary taurine-conjugated BA and has been shown to play a neuroprotective role in AD. It could prevent β-amyloid induced mitochondrial apoptosis by inhibiting oxygen-radical production (Amaral et al., 2009), reducing endoplasmic reticulum stress (Amaral et al., 2009), and stabilizing the unfolded protein responses (Ramalho et al., 2008). In addition, such neuroprotective effect of TUDCA has been extended to other neurological disorders, including Huntington's disease (Keene et al., 2002), Parkinson's

disease(Duan et al., 2002), and amyotrophic lateral sclerosis (Elia et al., 2016). Furthermore, pretreatment with TUDCA has been demonstrated to be effective in preventing cognitive impairment (Lo et al., 2013) and reducing amyloid deposition through the regulation of lipid-metabolism mediators (such as α2−macroglubulin, connective tissue growth factor, apolipoprotein E and its binding protein) in APP/PS1 mouse (Nunes et al., 2012). The decreased levels of taurine-conjugated primary BAs observed in transgenic female AD mice supported their neuroprotective effects in preventing amyloid plaques in AD (Figure 3). It is interesting to note that we observed the decreased levels of taurine conjugated BAs, but not glycine conjugates. This is because that the taurine conjugation is the preferred form in rodents (Falany et al., 1994, Falany et al., 1997).

Iso-DCA is a secondary BA and is produced by *Ruminococcus gnavus* from CA through gut microbiota (Figure 4) (A Sloan Devlin and Fischbach, 2015). Compared to other secondary BAs, iso-BAs is more easily absorbed by enterohepatic circulation because of their physicochemical properties with hydrogen bonding sites (Hamilton et al., 2007). Both iso-DCA and DCA are known to disrupt membrane integrity (A Sloan Devlin and Fischbach, 2015). Furthermore, increased levels of serum DCA and ratio of DCA:CA reflected changes in gut microbiome by converting primary into secondary BAs, which is consistent with study of AD population (MahmoudianDehkordi et al., 2019). In the current investigation, we observed increased levels of iso-DCA in liver extracts of female AD mice, which implied that the severity of female AD mice could be due to the cytotoxic impairment of iso-DCA, which in turn could be contributed by gut microbial biotransformation.

We also observed the decreased levels of T-β-MCA in jejunal content and increased levels of TDCA in liver extracts of AD male mice compared to their wild-type counterparts. Such gender-specific variations are also found in lipid metabolism of plasma and liver extracts in

male AD mice from our previous study (Wu et al., 2016). However the underlying reason of such gender differences in BA profiles remain to be elucidated.

In conclusion, we explored complex BA profiles of several compartments in enterohepatic circulation in an APP/PS1 double mutations induced AD mouse model. BA profiles in AD mice were gender dependent. The alterations of BAs were mainly found in jejunal contents, plasma and liver extracts of female AD mice, which manifested in the decreased levels of TUDCA and other taurine conjugated primary BAs. The severity of AD presented in female mice could be due to the reduced protective effects associated with the diminished levels of taurine conjugated BAs. On the contrary, increased levels of iso-DCA, produced by gut microbiota and known as a toxic BA, in female AD mice implied the cytotoxic impairment associated with AD *via* gut microbial biotransformation. Our results demonstrated that BAs profiles could be indicative of the interactions between host and gut microbiota and that monitoring the plasma BA profiles has important potentials for the surveillance of the neurological diseases. Most importantly, our study highlighted the gender specific BA profiles in AD.

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Author contributions

J.F. Wu performed animal experiments, conducted data analysis and contributed to manuscript writing. X.H. Zhu contributed for data analysis. Z.L. Chen and H. Lin developed an optimized LC-MS/MS method for bile acids. H.R.T contributed to the design of study. Y.L.W. contributed to the design of study, data analysis, interpretations and manuscript preparation.

Additional information

Competing interests: The authors declare no conflict of interest exists.

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Figure legends:

Figure 1. Relative BA concentrations detected from seven compartments in enterohepatic circulation, including four intestinal contents, feces, liver extracts and plasma of female transgenic mice and its wild type littermates. Values are mean of scaled (Log_e) data ± S.E.M. measures by UPLC-MS. Keys: FW: female wild-type group, FT: female transgenic group.

Figure 2. Concentrations of BAs in jejunal contents between transgenic mice and their comparable wild type counterparts. (A) female (B) male. Values are means ± S.E.M Statistics: *p<0.05. Keys: FW: female wild-type group, FT: female transgenic group, MW: male wild-type group, MT: male transgenic group.

Figure 3. Concentrations of BAs between transgenic AD mice and their comparable wild type counterparts in liver extracts (A, B) and plasma samples (C, D). Values are means ± S.E.M Statistics: *p<0.05, **p<0.01. Keys: FW: female wild-type group, FT: female transgenic group, MW: male wild-type group, MT: male transgenic group.

Figure 4. Summarized alterations of BA metabolism induced by double APP/PS1 transgenic mutations in mice. Metabolite levels shown in green and blue are indicative of increase and decrease respectively for the compounds in transgenic AD female mice when comparing to the control female mice.

Tables:

Table 1. The nomenclatures and abbreviations for the measured bile acids with categorization information.

Table 2. Concentration of bile acids in different biological matrices of female APP/PS1 transgenic mice and their wildtype littermates.

Table 3. Concentration of bile acids in different biological matrices of male APP/PS1 transgenic mice and their wildtype littermates.