

The influence of chronic L-carnitine supplementation on the formation of preneoplastic and atherosclerotic lesions in the colon and aorta of male F344 rats

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Abstract L-Carnitine, a key component of fatty acid oxidation, is nowadays being extensively used as a nutritional supplement with allegedly “fat burning” and performance-enhancing properties, although to date there are no conclusive data supporting these claims. Furthermore, there is an inverse relationship between exogenous supplementation and bioavailability, i.e., fairly high oral doses are not fully absorbed and thus a significant amount of carnitine remains in the gut. Human and rat enterobacteria can degrade unabsorbed L-carnitine to trimethylamine or trimethylamine-*N*-oxide, which, under certain conditions, may be transformed to the known carcinogen *N*-nitrosodimethylamine. Recent findings indicate that trimethylamine-*N*-oxide might also be involved in the development of atherosclerotic lesions. We therefore investigated whether a 1-year administration of different L-carnitine concentrations (0, 1, 2 and

5 g/l) via drinking water leads to an increased incidence of preneoplastic lesions (so-called *aberrant crypt foci*) in the colon of Fischer 344 rats as well as to the appearance of atherosclerotic lesions in the aorta of these animals. No significant difference between the test groups regarding the formation of lesions in the colon and aorta of the rats was observed, suggesting that, under the given experimental conditions, L-carnitine up to a concentration of 5 g/l in the drinking water does not have adverse effects on the gastrointestinal and vascular system of Fischer 344 rats.

Keywords L-Carnitine · Trimethylamine · Aberrant crypt foci · F344 rat · Atherosclerosis

Introduction

L-Carnitine is a key component of the so-called carnitine shuttle, a multienzyme transport system that is required to transfer activated long-chain fatty acids (acyl-CoAs) into the mitochondrial matrix, where they are degraded via β -oxidation (Violante et al. 2013). In the course of this process, L-carnitine is conjugated to acyl-CoAs by carnitine palmitoyltransferase 1 (CPT1) yielding acylcarnitines, which are then transported to the inner mitochondrial compartment by the carnitine acylcarnitine translocase (CACT) in exchange for free carnitine (Houten and Wanders 2010). Thereafter, carnitine palmitoyltransferase 2 (CPT2) retransforms acylcarnitines to acyl-CoA esters, which are then degraded to acyl-CoA subunits, thus generating substrates for the citric acid cycle and reducing equivalents for the electron transport chain (Houten and Wanders 2010).

Although L-carnitine is present in plants, the main nutritional sources for humans are foodstuffs of animal origin (Mitchell 1978). Depending on dietary habits, daily intake

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from food sources ranges from <0.16 to 2.4 mg/kg body weight (bw), the bioavailability being generally lower in humans that regularly consume a diet high in L-carnitine (e.g., abundant consumption of red meat) and even lower when high amounts of this compound are supplemented exogenously (Harper et al. 1988; Rebouche 2004; Sahajwalla et al. 1995). The body concentration of L-carnitine is tightly regulated by an equilibrium between endogenous synthesis (from lysine and methionine), renal reabsorption, and dietary L-carnitine supply, the latter especially influencing the renal clearance rate (Evans and Fornasini 2003; Jeukendrup et al. 1998; Rebouche 2004). Therefore, oral or intravenous dosages above a certain basal or physiological “threshold” level lead to an increased L-carnitine elimination and diminished uptake (Evans and Fornasini 2003; Rebouche and Seim 1998). Although the mechanisms of the intestinal absorption of L-carnitine have not yet been fully elucidated, they most likely involve carrier-mediated transport as well as passive diffusion, the latter being the more important intake route for non-dietary (i.e., high) carnitine concentrations (Evans and Fornasini 2003; Li et al. 1992). Active renal reabsorption, intestinal uptake and tissue distribution of L-carnitine are mediated by so-called carnitine/organic cation transporters (OCTN), two of them (OCTN1 and OCTN2) having been identified in humans (Tamai 2013). Rare mutations in the *SLC22A5* gene encoding for OCTN2 lead to systemic carnitine shortage and consequently to the development of a primary carnitine deficiency (PCD), which in most cases manifests itself clinically in form of a hypoketotic hypoglycemic encephalopathy as well as disorders of the heart and skeletal muscle (Erguven et al. 2007; Lahjouji et al. 2001). In contrast, the clinically less severe secondary carnitine deficiency (SCD) is caused by organ (e.g., kidney or liver) or metabolic disorders (e.g., impaired fatty acid metabolism), carnitine malabsorption, malnutrition as well as pharmacological treatment (Erguven et al. 2007; Flanagan et al. 2010). The treatment of these disorders, especially in the case of PCD, consists in the daily supplementation of L-carnitine in doses (100–400 mg/kg bw or 990 mg 2–3 times/day) adapted to the patients’ plasma level (Bain et al. 2006; Longo et al. 2006).

Since the early 1980s, L-carnitine is also being extensively advertised and used by athletes, bodybuilders or even obese individuals as a nutritional supplement with allegedly “fat burning” and performance-enhancing properties (Jeukendrup et al. 1998), although to date there are no conclusive data supporting these claims neither in rats (Eder 2000; Melton et al. 2005; Saldanha Aoki et al. 2004) nor in humans (Barnett et al. 1994; Brass 2000; Cerretelli and Marconi 1990; Grunewald and Bailey 1993; Jeukendrup and Randell 2011; Jeukendrup et al. 1998; Vukovich et al. 1994). Taking into account the inverse relationship between

exogenous supplementation and bioavailability, one must conclude that, when fairly high oral doses are given, a significant amount of L-carnitine would remain in the gut. The unabsorbed compound can be partially degraded to trimethylamine (TMA) or γ -butyrobetaine by enterobacteria in the gut lumen of rats and, to a greater extent, humans (Koeth et al. 2013; Rebouche and Chenard 1991; Rebouche et al. 1984; Zhang et al. 1999). After absorption, TMA is oxidized to trimethylamine-*N*-oxide (TMNO) by hepatic flavin monooxygenases (Baker and Chaykin 1962; Bennett et al. 2013; Koeth et al. 2013): In addition to that, and following L-carnitine supplementation, TMNO can be directly produced in the gut by the gastrointestinal microbiota (Koeth et al. 2013). In an acidic environment and in the presence of nitrite ions, i.e., under conditions which prevail in the upper gastrointestinal tract, the known carcinogen *N*-nitrosodimethylamine (NDMA) can be formed from these amine precursors (Bain et al. 2005; Lijinsky et al. 1972; Loh et al. 2011; Tricker and Preussmann 1991). Additionally, bacterial metabolism can also lead to NDMA formation from amines such as TMA and dimethylamine (Maduagwu and Bassir 1979). Consumption of L-carnitine in doses that are not completely absorbed might thus enhance bacterial NDMA production in the colon and consequently contribute to colorectal tumor formation, as has been shown by Knekt et al. (1999) and Loh et al. (2011) for dietary NDMA.

Therefore, we investigated whether a chronic administration of different L-carnitine concentrations via drinking water leads to an increased number of *aberrant crypt foci* (ACF), which are considered preneoplastic lesions associated with colorectal cancer formation (Bird 1995), in the colon of male Fischer 344 rats. As a recent study by Koeth et al. (2013) showed that TMNO resulting from L-carnitine supplementation promotes atherosclerosis in mice, we additionally examined its influence on the occurrence of atherosclerotic lesions in the aorta of the above-mentioned rats.

Materials and methods

Animals, housing and diet

Eighty male Fischer 344 DuCrI rats (F344 rats) were purchased at 5–6 weeks of age (100–120 g bw) from Charles River (Sulzfeld, Germany) and housed in type IV polycarbonate cages (EHRET, Emmendingen, Germany). The cages were placed in airflow cabinets (Uni Protect; EHRET) operated in a positive pressure mode (50 Pa) and providing a temperature of 21–23 °C, a relative humidity of 50–60 %, a maximum light intensity of 45 lux, 15–20 air shifts per hour as well as a 12/12 h day and night cycle. The bedding consisted of poplar granules (LIGNOCEL[®] Select; JRS,

Rosenberg, Germany), which were changed once a week, and the diet was a standard pelleted rodent maintenance diet (cat. nr. 1324; see online resource 1 “Specifications of the animal feed” for details on feed composition) purchased from Altromin (Lage, Germany). The animals in each cage had access to tunnel housing made of red polycarbonate (BIOSCAPE, Castrop-Rauxel, Germany) and certified carcinogen and toxicant-free aspen rods (ABEDD[®] Lab&Vet Service, Vienna, Austria) as enrichment.

Experimental design and procedure

Upon arrival, littermates were randomly assigned to one of four test groups consisting of 20 animals each and housed pairwise in each cage to minimize distress during the whole experimental period of 58 weeks. Animals in the control group (group 1) received drinking (tap) water without any supplementation. Water for groups 2, 3 and 4 was supplemented with 1, 2 or 5 g L-carnitine/l for 52 weeks, respectively. L-Carnitine (Carnipure[™]; Lonza, Basel, Switzerland) with a purity of 99.5–99.9 % was purchased from Denk Ingredients (Munich, Germany). Because of a sialodacryoadenitis virus (SDAV) infection (see results), the L-carnitine treatment was started in week 6 upon arrival after an acclimatization and recovery period of 5 weeks. To avoid microbial contamination, drinking water was autoclaved before carnitine supplementation and changed twice a week. In the course of water changes, water consumption was recorded, while the weight of the animals was assessed once a week. Moreover, the stability of L-carnitine in the water was assessed by liquid chromatography/mass spectrometry (LC–MS) for a period of 7 days under experimental conditions (see online resource 1 “Assessment of L-carnitine stability” for details). After the 52-week administration period, individual rats were anaesthetized by CO₂ (6 L/min flush) and decapitated. Blood was immediately collected for further analysis and the gastrointestinal tract entirely removed and processed as previously described (Nicken et al. 2012). Briefly, the colon was removed, washed with phosphate buffered saline (PBS) and opened longitudinally. Thereafter, the tissues were fixed in formalin (Roti[®]-Histofix 4 %; Carl Roth, Karlsruhe, Germany), stained with methylene blue solution (0.1 % w/v in PBS) and ACF formation was assessed using a stereomicroscope (SZX16; Olympus, Hamburg, Germany). Additionally, the kidneys, liver and spleen were removed and weighed. For histopathologic examination, heart, thoracic aorta and liver were fixed in 10 % neutral buffered formalin. Organ trimming was performed in accordance with the Registry of Industrial Toxicology Animal-data (RITA) and North American Control Animal Database (NACAD) guidelines for organ sampling and trimming in rats and mice (Morawietz et al. 2004; Ruehl-Fehlert et al. 2003), followed by

embedding in paraffin wax, sectioning at 2 µm thickness, and staining with hematoxylin and eosin (HE).

Imaging and illustrations

Micrographs of representative lesions were obtained using an Olympus BX51 microscope equipped with a DP72 12.8 megapixel digital color camera and cellSens Standard v. 1.7.1 software (Olympus Corp., Tokyo, Japan). Figures were further processed with Adobe[®] Photoshop[®] v. 7.0 (Adobe Systems, Inc., San Jose, CA, USA), thereby adjusting contrast and brightness, if necessary.

Assessment of the NDMA concentration in the urine of the experimental animals

Urine was collected in the next-to-last week of the study by housing 16 animals (4 from each group) individually in metabolic cages (TECNIPLAST, Hohenpeißenberg, Germany) for 24 h. Gathered urine samples (5–10 ml) were stored in 50-ml tubes (Greiner Bio-One, Frickenhausen, Germany) protected from light at –80 °C until analysis. Sample extraction was performed using Supelclean coconut charcoal SPE tubes (Sigma-Aldrich, Schnellendorf, Germany) based on the U.S. Environmental Protection Agency method 521 for the detection of nitrosamines in drinking water (Munch and Bassett 2004). d₆-NDMA (Restek, Bad Homburg, Germany) was added to the urine samples as internal standard prior to sample preparation. The nitrosamines were eluted from the SPE tubes using methylene chloride (VWR, Leuven, Belgium) and concentrated under a gentle stream of nitrogen at 35 °C to a volume of approximately 0.5 ml. An aliquot of 5 µl was injected into the GC–MS. As residue-free urine was not available, the method validation was performed by using synthetic human urine (Synthetic Urine, Nussdorf, Germany) spiked with NDMA (Restek). The samples were analyzed on an Agilent 7890A gas chromatograph (Waldbronn, Germany) coupled to an Agilent 5975C mass selective detector (MSD) applying the following parameters: column: Agilent DB-WAX (polyethylene glycol, 30 m; 0.25 mm i.d.; 0.5 µm film thickness); carrier gas: helium, 1.9 ml/min, constant flow; oven temperature program: 1 min 35 °C, +20 °C/min, 1 min 200 °C, backflush 3 min 200 °C; programmed temperature vaporization on an Agilent multimode inlet: inlet temperature program: 0.06 min 37 °C, +600 °C/min, 5 min 240 °C; vent flow 100 ml/min at 0.34 bar; ionization: 70 eV, EI, SIM mode (*m/z*: 80, 74, 42).

Statistical analysis

Statistical analysis of the data was performed with Prism v. 6.04 (GraphPad Software, Inc., La Jolla, CA, USA). The

Table 1 Physiological data of the experimental animals

	Group 1 (0 g/l)	Group 2 (1 g/l)	Group 3 (2 g/l)	Group 4 (5 g/l)
Number of animals	19	19	19	20
Starting body weight (g) ^a	105.1 ± 7.9	102.4 ± 7.0	101.9 ± 6.9	101.6 ± 6.9
Final body weight (g) ^a	410.5 ± 19.6	408.5 ± 14.6	413.2 ± 14.1	414.5 ± 18.3
Kidney weight (g)* ^a	1.27 ± 0.08	1.26 ± 0.07	1.28 ± 0.1	1.30 ± 0.09
Liver weight (g) ^b	11.4 ± 1.0	11.4 ± 0.9	11.6 ± 0.8	11.9 ± 0.9
Spleen weight (g) ^b	0.82 ± 0.1	0.83 ± 0.08	0.84 ± 0.06	0.84 ± 0.08
Water uptake (ml/day/animal) ^{▲^a}	18.1 ± 1.6	18.1 ± 2.1	18.3 ± 1.2	18.8 ± 1.8 ^{α,β}

Where applicable, values are shown as mean ± standard deviation

* Weight of one kidney

▲ Calculated on the basis of the mean of the weekly water consumption of two animals/cage

^a Kruskal–Wallis test followed by Dunn’s post hoc analysis

^b One-way ANOVA followed by Tukey’s post hoc analysis

^α Significantly different ($p < 0.01$) when compared to group 1

^β Significantly different ($p < 0.001$) when compared to group 2

Shapiro–Wilk normality test was used to assess probability distribution of the datasets. Normally distributed sets were subjected to a one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test, while non-normally distributed data or data with too few independent experiments to perform a Shapiro–Wilk test (analysis of NDMA levels in rat urine) were subjected to a Kruskal–Wallis test followed by Dunn’s post hoc comparison. The relationship between the frequencies of pathohistological findings and L-carnitine treatment was analyzed by means of Pearson’s chi-squared test. Statistical significance was considered if $p \leq 0.05$.

Results

Clinical observations

In the first week upon arrival, the animals showed signs of an SDAV infection. SDAV is a relatively common and rat-specific coronavirus with high morbidity and very low-to-no mortality (Gaillard and Clifford 2000; Jacoby and Gaertner 2006). The infection was relatively silent, the most prominent clinical symptom being sneezing followed by red-colored nasal discharge.

Experimental findings

In groups 1, 2 and 3, one animal had to be euthanized before the completion of the study. All other animals completed the study in good general condition. No statistically significant differences were observed between the groups regarding the final body weight, all animals weighing ~410 g at the end of the study (Table 1, “Final bw”).

Similarly, the final weight of the various organs sampled from the different animals did not differ across the groups in a significant manner (Table 1, “Kidney, liver and spleen weight”). Interestingly, the rats in the highest dose group (group 4) drank significantly more water than the animals in groups 1 (control) and 2 (lowest dose; Table 1, “Water uptake”). Based on average water consumption of 18.3 ml/rat/day (Table 1, “Water uptake”) and an average body weight of 260 g/rat (Table 1; approximate average between starting and final body weight), animals in groups 2, 3 and 4 ingested 70.4, 140.8 and 351.9 mg L-carnitine/kg bw/day, respectively. Based on a formula published by Reagan-Shaw et al. (2008) and using standard conversion factors published by the U.S. Food and Drug Administration (Center for Drug Evaluation and Research 2005), these dosages would equal to a human equivalent dose (HED) of ~11.4, ~22.8 and ~57.1 mg/kg bw, respectively.

Regarding ACF formation and ACF multiplicity (crypts/ACF), there was no statistically significant difference between the four groups tested (Tables 2, 3)

Histopathological alterations in the aorta were only seen in one animal of the control group, showing mild focal degenerative changes in the media accompanied by a mild infiltration of macrophages and mineralization (Fig. 1a; Table 3). Histopathological examination of the heart revealed a mild multifocal chronic lymphohistiocytic myocarditis with myocardial degeneration and fibrosis in about 50 % of the rats, but no statistically significant difference between the groups was observed (Fig. 1b; Table 3). Moreover, all animals displayed a variable degree of bile duct hyperplasia (Fig. 1c), and a high number of animals showed a mild-to-moderate multifocal acute to subacute

Table 2 Number of ACF/animal and number of crypts/ACF

	Group 1 (0 g/l)	Group 2 (1 g/l)	Group 3 (2 g/l)	Group 4 (5 g/l)
Number of animals with ACFs ^a	1/19 (5.3)	3/19 (15.8)	2/19 (10.5)	0/20 (0)
Localization of ACFs	Middle part of the colon	Middle (2) and distal (1) part of the colon	Middle part of the colon	–
ACFs/animal ^b	0 ± 0–1	0 ± 0–1	0 ± 0–1	0 ± 0–0
Crypts/ACF	2 ± 2–2	2 ± 2–3	2.5 ± 2–3	0 ± 0–0

Where applicable, values are shown as median ± range

^a Number of animals with ACFs/total number of animals analyzed, frequency (%) in parentheses

^b Kruskal–Wallis test followed by Dunn's post hoc analysis

Table 3 Pathohistological findings in the aorta, heart and liver of the animals

	Group 1 (0 g/l)	Group 2 (1 g/l)	Group 3 (2 g/l)	Group 4 (5 g/l)
Focal degenerative changes in the aorta ^a	1/19 (5.3)	0/19 (0)	0/19 (0)	0/20 (0)
Lymphohistiocytic infiltrations in the heart (myocarditis) ^a	12/19 (63.2)	11/19 (57.9)	9/19 (47.4)	8/20 (40.0)
Myocardial degeneration ^a	12/19 (63.2)	6/19 (31.6)	10/19 (52.6)	10/20 (50.0)
Myocardial fibrosis ^a	11/19 (57.9)	5/19 (26.3)	10/19 (52.6)	10/20 (50.0)
Suppurative and necrotizing hepatitis ^a	15/19 (78.9)	13/19 (68.4)	16/19 (84.2)	18/20 (90.0)
Bile duct hyperplasia ^a	19/19 (100)	19/19 (100)	19/19 (100)	20/20 (100)

All pathohistological findings analyzed with Pearson's chi-squared test

^a Number of animals with the mentioned alteration/total number of animals analyzed, frequency (%) in parentheses

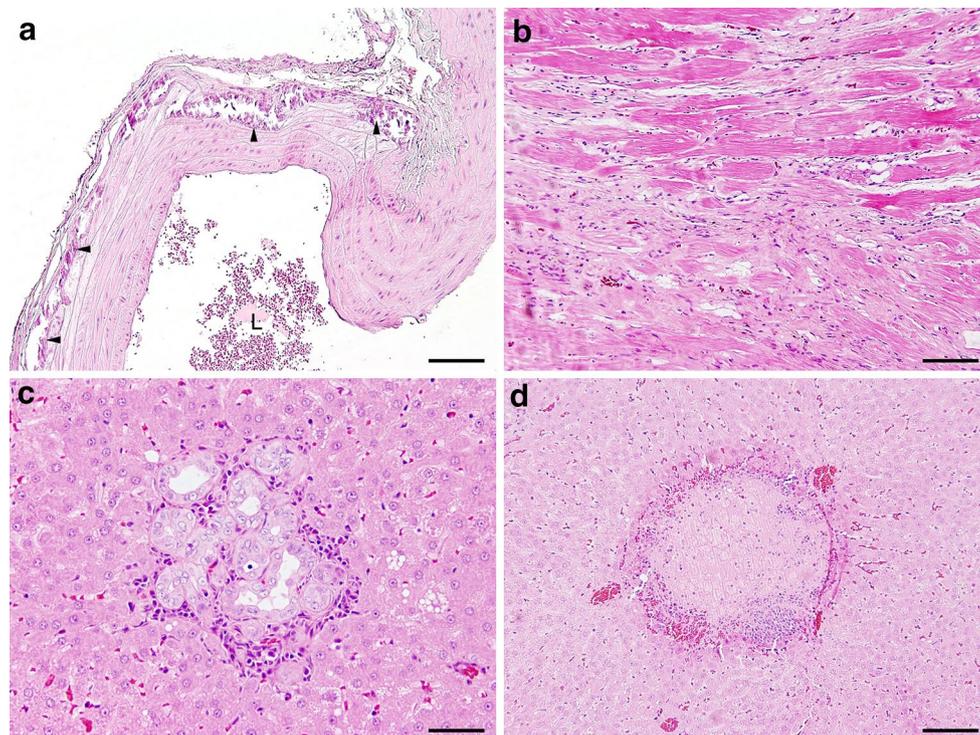


Fig. 1 **a** Aorta of a control animal: mild focal degenerative changes in the media accompanied by a mild infiltration of macrophages and mineralization (*arrowheads*; *L* lumen). **b** Heart of an animal of group 4: mild multifocal chronic lymphohistiocytic myocarditis with myocardial degeneration and fibrosis. **c** Liver of a control ani-

mal: mild bile duct hyperplasia, associated with mild lymphohistiocytic infiltration. **d** Liver of a control animal: focal suppurative and necrotizing hepatitis. HE. Scale bars **a**, **b**, **d** 100 μ m, scale bar **c** 50 μ m

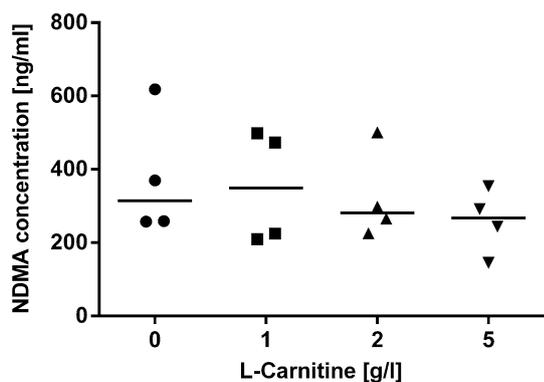


Fig. 2 NDMA concentrations in the urine of the experimental animals collected in the next-to-last week of the study. Shown is the median as well as NDMA levels of each individual animal/group (mean of three measurements/sample). The dataset was subjected to a Kruskal–Wallis test followed by Dunn’s post hoc comparison

suppurative and necrotizing hepatitis (Fig. 1d), without statistically significant differences between the groups (Table 3). Except for a hepatocellular carcinoma in one animal of group 1, no tumors were observed within the livers of the remaining animals.

No significant differences regarding the NDMA content in the urine of the animals was observed between the test groups (Fig. 2). Interestingly, animals receiving 2 or 5 g/l L-carnitine (groups 3 and 4) seem to excrete the lowest amount of NDMA, median concentrations reaching 281.9 ng/ml and 267.6 ng/ml, respectively (group 1: 314.2 ng/ml; group 2: 348.8 ng/ml). However, it has to be noted that the differences are not statistically significant and that the amount of urinary NDMA strongly varies between the animals tested in each group (Fig. 2).

Discussion

The final mean body weights of the rats recorded during the course of this study correspond to weights measured in untreated F344 rats of the same age (Solleveld et al. 1984). In contrast, the water uptake of the rats in this study was somewhat reduced when compared to the water uptake of laboratory rats in general (Hofstetter et al. 2006). Although rats administered 5 g/l L-carnitine drank significantly more water than animals in groups 1 and 2, this finding can be considered as biologically irrelevant, and it is questionable whether it is actually related to L-carnitine supplementation. F344 rats have extensively been used in long-term (i.e., 2 years-long) carcinogenicity studies (Dinse et al. 2010; Solleveld et al. 1984) and are characterized by an extremely low spontaneous incidence rate (0.1–0.6 %) of neoplasms of the small and large intestine (Haseman et al. 1998), rendering them particularly useful for the

investigation of putative colon carcinogens. However, ACFs seem to spontaneously develop in the colon of F344 rats with a fairly high incidence (40–60 %), even in animals killed at an earlier age than those in the present study (Furukawa et al. 2002; Tanakamaru et al. 2001). With an overall frequency of 7.7 % and no statistically significant difference in the number of ACFs/animal between the testing groups, ACF formation in this study is considered to be spontaneous and not related to L-carnitine supplementation. In addition, the data obtained in the course of this study show that the administration of different L-carnitine dosages does not lead to increased amounts of NDMA being excreted via the urine when compared to untreated animals, a fact which might furthermore explain the low ACF incidence. Even though there was no difference between the test groups, it should be noted that a “basal” NDMA level (376.2 ± 169.8 ng/ml on average) was nevertheless observable in the urine of the animals in group 1. This might be the result of its endogenous formation or of a contamination of unknown etiology (Kraft et al. 1981; Tricker and Preussmann 1991; Vermeer et al. 1998).

Regarding the possible influence of the SDAV infection on the outcome of the study at hand, it should be mentioned that the repair processes in the affected tissues (respiratory tract, eye, salivary and lacrimal glands) begin 5–7 days post infection and in the case of the salivary and lacrimal glands are completed after about 21 days (Jacoby and Gaertner 2006; Percy et al. 1988). Since SDAV infection occurred at the very beginning of the study and the animals had 3–4 weeks to recover, we do not suspect any major effect of the virus on the outcome of this study, especially since SDAV is not known to interfere with colon tumorigenesis or heart-related pathologies (Jacoby and Gaertner 2006). The latter statement as well as the fact that immunocompetent animals develop immunity and recover relatively fast with barely any sequelae (Jacoby and Gaertner 2006) led to the decision, not to kill the entire colony and to continue the experiment.

Particularly, male rats develop a plethora of pathologic changes with increasing age, the most common being spontaneous tumors, chronic nephropathy and lesions of the cardiovascular system (Coleman et al. 1977; King and Russell 2006). The pathologic findings related to the cardiovascular system described in this study clearly fall in this category. Focal myocardial degeneration in conjunction with inflammation (lymphohistiocytic infiltrations) followed by myocardial fibrosis have been described in aged F344 rats as well as other rat strains in incidences comparable to the ones reported herein, especially when the animals were fed ad libitum (Blankenship and Skaggs 2013; Coleman et al. 1977; Goodman et al. 1979; Hall et al. 1992; Keenan et al. 1995a, b; Maeda et al. 1985). In this context, the left ventricular papillary muscle, which was the primarily affected

location in numerous animals (data not shown), is reported to be a preferred site for these lesions (Percy and Barthold 2008). Additionally, aging rats commonly exhibit hyperplastic bile ducts (King and Russell 2006). According to Coleman et al. (1977), 75 % of approximately 12-month old F344 rats show bile duct hyperplasia, a finding which contrasts the incidence of 100 % reported in this study, although, still in accordance with the same source, similar incidences were reported in significantly older animals (>18 months). In contrast, control F344 rats used in 2-year carcinogenicity studies of the U.S. National Institutes of Health Carcinogenesis Testing Program only marginally suffered from bile duct hyperplasia, 24.5 % of male and 12.5 % of female F344 rats being affected (Goodman et al. 1979). The frequency of multifocal suppurative and necrotizing liver lesions observed in this study (68.4–90.0 %) was distinctly above the relatively low incidence of spontaneous necrotizing processes previously described in aged male F344 rats (approx. 6,9 %; Hall et al. 1992). Although we were unable to identify characteristic viral, bacterial, mycotic or parasitic structures within the liver lesions employing routine and special histological staining methods (Gram stain, periodic acid Schiff-reaction, and Grocott silver stain; data not shown), we cannot rule out the possibility that the animals were infected with agents such as *Clostridium piliforme* (Tyzzer's disease), *Salmonella spp.* or *Corynebacterium kutscheri* (Percy and Barthold 2008; Thoolen et al. 2010). However, since all test groups were equally affected, this finding is clearly not related to the L-carnitine treatment. Additionally, it should be considered that these lesions were mainly of mild, sub-clinical grade and acute in character and therefore might have developed only in the last days (or the last week) of the study, thus rendering an influence on ACF formation or the onset of cardiovascular lesions unlikely.

No definitive (sub-)intimal atherosclerotic lesions were observed in the aortas of the animals. The degenerative changes observed in the aorta of one control rat were interpreted as an incidental finding of unknown etiology and are in agreement with the previously reported rare spontaneous lesions described in old F344 rats (King and Russell 2006). Since this finding concerns only one animal in the control group, a carnitine-related cause can be excluded. Additionally, it should be noted that rats, except for specially bred strains, generally do not develop atherosclerosis (King and Russell 2006; Moghadasian 2002). This fact together with differences in the composition of the gut microbiota, the metabolism of L-carnitine as well as the amount (1.3 g/l) of L-carnitine given to the apolipoprotein E-knockout mice might explain the divergent results of the study at hand to that conducted by Koeth et al. (2013) regarding the formation of atherosclerotic lesions.

In conclusion, this study provides evidence that the daily administration of L-carnitine in concentrations of 70.4, 140.8 and 351.9 mg/kg bw/day for 1 year does not lead to an adverse effect in the colon or cardiovascular system of male F344 rats.

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References

- Bain MA, Fornasini G, Evans AM (2005) Trimethylamine: metabolic, pharmacokinetic and safety aspects. *Curr Drug Metab* 6(3):227–240
- Bain MA, Milne RW, Evans AM (2006) Disposition and metabolite kinetics of oral L-carnitine in humans. *J Clin Pharmacol* 46(10):1163–1170
- Baker JR, Chaykin S (1962) The biosynthesis of trimethylamine-*N*-oxide. *J Biol Chem* 237:1309–1313
- Barnett C, Costill DL, Vukovich MD et al (1994) Effect of L-carnitine supplementation on muscle and blood carnitine content and lactate accumulation during high-intensity sprint cycling. *Int J Sport Nutr* 4(3):280–288
- Bennett BJ, de Aguiar Vallim TQ, Wang Z et al (2013) Trimethylamine-*N*-oxide, a metabolite associated with atherosclerosis, exhibits complex genetic and dietary regulation. *Cell Metab* 17(1):49–60
- Bird RP (1995) Role of aberrant crypt foci in understanding the pathogenesis of colon cancer. *Cancer Lett* 93(1):55–71
- Blankenship B, Skaggs H (2013) Findings in historical control Harlan RCCHan™: WIST rats from 4-, 13-, 26-week studies. *Toxicol Pathol* 41(3):537–547
- Brass EP (2000) Supplemental carnitine and exercise. *Am J Clin Nutr* 72(2 Suppl):618S–623S
- Center for Drug Evaluation and Research (2005) Guidance for industry—estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers. In: Information DoD (ed). U.S. Food and Drug Administration, Rockville, MD, USA
- Cerretelli P, Marconi C (1990) L-carnitine supplementation in humans. The effects on physical performance. *Int J Sports Med* 11(1):1–14
- Coleman GL, Barthold W, Osbaldiston GW, Foster SJ, Jonas AM (1977) Pathological changes during aging in barrier-reared Fischer 344 male rats. *J Gerontol* 32(3):258–278
- Dinse GE, Peddada SD, Harris SF, Elmore SA (2010) Comparison of NTP historical control tumor incidence rates in female Harlan Sprague Dawley and Fischer 344/N rats. *Toxicol Pathol* 38(5):765–775
- Eder K (2000) L-carnitine supplementation and lipid metabolism of rats fed a hyperlipidaemic diet. *J Anim Physiol Anim Nutr (Berl)* 83(3):132–140
- Erguven M, Yilmaz O, Koc S et al (2007) A case of early diagnosed carnitine deficiency presenting with respiratory symptoms. *Ann Nutr Metab* 51(4):331–334

- Evans AM, Fornasini G (2003) Pharmacokinetics of L-carnitine. *Clin Pharmacokinet* 42(11):941–967
- Flanagan JL, Simmons PA, Vehige J, Willcox MD, Garrett Q (2010) Role of carnitine in disease. *Nutr Metab (Lond)* 7:30
- Furukawa F, Nishikawa A, Kitahori Y, Tanakamaru Z, Hirose M (2002) Spontaneous development of aberrant crypt foci in F344 rats. *J Exp Clin Cancer Res* 21(2):197–201
- Gaillard ET, Clifford CB (2000) Common diseases. In: Krinke GJ (ed) *The laboratory rat*. Academic Press, London, pp 99–132
- Goodman DG, Ward JM, Squire RA, Chu KC, Linhart MS (1979) Neoplastic and nonneoplastic lesions in aging F344 rats. *Toxicol Appl Pharmacol* 48(2):237–248
- Grunewald KK, Bailey RS (1993) Commercially marketed supplements for bodybuilding athletes. *Sports Med* 15(2):90–103
- Hall WC, Ganaway JR, Rao GN et al (1992) Histopathologic observations in weanling B6C3F1 mice and F344/N rats and their adult parental strains. *Toxicol Pathol* 20(2):146–154
- Harper P, Elwin CE, Cederblad G (1988) Pharmacokinetics of intravenous and oral bolus doses of L-carnitine in healthy subjects. *Eur J Clin Pharmacol* 35(5):555–562
- Haseman JK, Hailey JR, Morris RW (1998) Spontaneous neoplasm incidences in Fischer 344 rats and B6C3F1 mice in two-year carcinogenicity studies: a national toxicology program update. *Toxicol Pathol* 26(3):428–441
- Hofstetter J, Suckow MA, Hickman DL (2006) Morphophysiology. In: Suckow MA, Weisbroth SH, Franklin CL (eds) *The laboratory rat*, 2nd edn. Academic Press, Burlington, pp 93–125
- Houten SM, Wanders RJ (2010) A general introduction to the biochemistry of mitochondrial fatty acid beta-oxidation. *J Inher Metab Dis* 33(5):469–477
- Jacoby RO, Gaertner DJ (2006) Viral disease. In: Suckow MA, Weisbroth SH, Franklin CL (eds) *The laboratory rat*, 2nd edn. Academic Press, Burlington, pp 423–451
- Jeukendrup AE, Randell R (2011) Fat burners: nutrition supplements that increase fat metabolism. *Obes Rev* 12(10):841–851
- Jeukendrup AE, Saris WH, Wagenmakers AJ (1998) Fat metabolism during exercise: a review—part III: effects of nutritional interventions. *Int J Sports Med* 19(6):371–379
- Keenan KP, Soper KA, Hertzog PR et al (1995a) Diet, overfeeding, and moderate dietary restriction in control Sprague–Dawley rats: II. Effects on age-related proliferative and degenerative lesions. *Toxicol Pathol* 23(3):287–302
- Keenan KP, Soper KA, Smith PF, Ballam GC, Clark RL (1995b) Diet, overfeeding, and moderate dietary restriction in control Sprague–Dawley rats: I. Effects on spontaneous neoplasms. *Toxicol Pathol* 23(3):269–286
- King WW, Russell SP (2006) Metabolic, traumatic, and miscellaneous diseases. In: Suckow MA, Weisbroth SH, Franklin CL (eds) *The laboratory rat*, 2nd edn. Academic Press, Burlington, pp 513–546
- Knekt P, Jarvinen R, Dich J, Hakulinen T (1999) Risk of colorectal and other gastro-intestinal cancers after exposure to nitrate, nitrite and N-nitroso compounds: a follow-up study. *Int J Cancer* 80(6):852–856
- Koeth RA, Wang Z, Levison BS et al (2013) Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. *Nat Med* 19(5):576–585
- Kraft PL, Skipper PL, Charnley G, Tannenbaum SR (1981) Urinary excretion of dimethylnitrosamine: a quantitative relationship between dose and urinary excretion. *Carcinogenesis* 2(7):609–612
- Lahjouji K, Mitchell GA, Qureshi IA (2001) Carnitine transport by organic cation transporters and systemic carnitine deficiency. *Mol Genet Metab* 73(4):287–297
- Li B, Lloyd ML, Gudjonsson H, Shug AL, Olsen WA (1992) The effect of enteral carnitine administration in humans. *Am J Clin Nutr* 55(4):838–845
- Lijinsky W, Keefer L, Conrad E, Van de Bogart R (1972) Nitrosation of tertiary amines and some biologic implications. *J Natl Cancer Inst* 49(5):1239–1249
- Loh YH, Jakszyn P, Luben RN, Mulligan AA, Mitrou PN, Khaw KT (2011) N-Nitroso compounds and cancer incidence: the European prospective investigation into cancer and nutrition (EPIC)-norfolk study. *Am J Clin Nutr* 93(5):1053–1061
- Longo N, Amat di San Filippo C, Pasquali M (2006) Disorders of carnitine transport and the carnitine cycle. *Am J Med Genet C Semin Med Genet* 142C(2):77–85
- Maduagwu EN, Bassir O (1979) Microbial nitrosamine formation in palm wine: in vitro N-nitrosation by cell suspensions. *J Environ Pathol Toxicol* 2(4):1183–1194
- Maeda H, Gleiser CA, Masoro EJ, Murata I, McMahan CA, Yu BP (1985) Nutritional influences on aging of Fischer 344 rats: II. Pathology. *J Gerontol* 40(6):671–688
- Melton SA, Keenan MJ, Stanciu CE et al (2005) L-carnitine supplementation does not promote weight loss in ovariectomized rats despite endurance exercise. *Int J Vitam Nutr Res* 75(2):156–160
- Mitchell ME (1978) Carnitine metabolism in human subjects. I. Normal metabolism. *Am J Clin Nutr* 31(2):293–306
- Moghadasian MH (2002) Experimental atherosclerosis: a historical overview. *Life Sci* 70(8):855–865
- Morawietz G, Ruehl-Fehlert C, Kittel B et al (2004) Revised guides for organ sampling and trimming in rats and mice—part 3: a joint publication of the RITA and NACAD groups. *Exp Toxicol Pathol* 55(6):433–449
- Munch JW, Bassett MV (2004) Version 1.0; document # EPA/600/R-05/054) Determination of nitrosamines in drinking water by solid phase extraction and capillary column gas chromatography with large volume injection and chemical ionization tandem mass spectrometry (MS/MS). U.S. Environmental Protection Agency, Cincinnati, Ohio
- Nicken P, Brauer N, Lampen A, Steinberg P (2012) Influence of a fat-rich diet, folic acid supplementation and a human-relevant concentration of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine on the induction of preneoplastic lesions in the rat colon. *Arch Toxicol* 86(5):815–821. doi:10.1007/s00204-012-0819-1
- Percy DH, Barthold SW (2008) *Rat pathology of laboratory rodents and rabbits*. Blackwell Publishing Professional, Oxford, pp 125–177
- Percy DH, Hayes MA, Kocal TE, Wojcinski ZW (1988) Depletion of salivary gland epidermal growth factor by sialodacryadenitis virus infection in the Wistar rat. *Vet Pathol* 25(3):183–192
- Reagan-Shaw S, Nihal M, Ahmad N (2008) Dose translation from animal to human studies revisited. *FASEB J* 22(3):659–661. doi:10.1096/fj.07-9574LSF
- Rebouche CJ (2004) Kinetics, pharmacokinetics, and regulation of L-carnitine and acetyl-L-carnitine metabolism. *Ann N Y Acad Sci* 1033:30–41
- Rebouche CJ, Chenard CA (1991) Metabolic fate of dietary carnitine in human adults: identification and quantification of urinary and fecal metabolites. *J Nutr* 121(4):539–546
- Rebouche CJ, Seim H (1998) Carnitine metabolism and its regulation in microorganisms and mammals. *Annu Rev Nutr* 18:39–61
- Rebouche CJ, Mack DL, Edmonson PF (1984) L-Carnitine dissimilation in the gastrointestinal tract of the rat. *Biochemistry* 23(26):6422–6426
- Ruehl-Fehlert C, Kittel B, Morawietz G et al (2003) Revised guides for organ sampling and trimming in rats and mice—part 1: a joint publication of the RITA and NACAD groups. *Exp Toxicol Pathol* 55(2–3):91–106

- Sahajwalla CG, Helton ED, Purich ED, Hoppel CL, Cabana BE (1995) Multiple-dose pharmacokinetics and bioequivalence of L-carnitine 330-mg tablet versus 1-g chewable tablet versus enteral solution in healthy adult male volunteers. *J Pharm Sci* 84(5):627–633
- Saldanha Aoki M, Rodriguez Amaral Almeida AL, Navarro F, Bicudo Pereira Costa-Rosa LF, Pereira Bacurau RF (2004) Carnitine supplementation fails to maximize fat mass loss induced by endurance training in rats. *Ann Nutr Metab* 48(2):90–94
- Solleveld HA, Haseman JK, McConnell EE (1984) Natural history of body weight gain, survival, and neoplasia in the F344 rat. *J Natl Cancer Inst* 72(4):929–940
- Tamai I (2013) Pharmacological and pathophysiological roles of carnitine/organic cation transporters (OCTNs: SLC22A4, SLC22A5 and SLC22A21). *Biopharm Drug Dispos* 34(1):29–44
- Tanakamaru Z, Mori I, Nishikawa A, Furukawa F, Takahashi M, Mori H (2001) Essential similarities between spontaneous and MeIQx-promoted aberrant crypt foci in the F344 rat colon. *Cancer Lett* 172(2):143–149
- Thoolen B, Maronpot RR, Harada T et al (2010) Proliferative and nonproliferative lesions of the rat and mouse hepatobiliary system. *Toxicol Pathol* 38(7 Suppl.):5S–81S
- Tricker AR, Preussmann R (1991) Carcinogenic *N*-nitrosamines in the diet: occurrence, formation, mechanisms and carcinogenic potential. *Mutat Res* 259(3–4):277–289
- Vermeer IT, Pachen DM, Dallinga JW, Kleinjans JC, van Maanen JM (1998) Volatile *N*-nitrosamine formation after intake of nitrate at the ADI level in combination with an amine-rich diet. *Environ Health Perspect* 106(8):459–463
- Violante S, Ijlst L, Te Brinke H et al (2013) Peroxisomes contribute to the acylcarnitine production when the carnitine shuttle is deficient. *Biochim Biophys Acta* 1831(9):1467–1474
- Vukovich MD, Costill DL, Fink WJ (1994) Carnitine supplementation: effect on muscle carnitine and glycogen content during exercise. *Med Sci Sports Exerc* 26(9):1122–1129
- Zhang AQ, Mitchell SC, Smith RL (1999) Dietary precursors of trimethylamine in man: a pilot study. *Food Chem Toxicol* 37(5):515–520