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Original article

# Nrf2 transcriptional activity in the mouse affects the physiological response to tribromoethanol

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ABSTRACT

Up to date, there is no information on the influence of 2,2,2-tribromoethanol (TBE; Avertin), a commonly used anaesthetic, on mice with impaired antioxidant capacity. We aimed to analyse the effect of a single dose of Avertin on anaesthesia duration time, inflammatory response, oxidative stress and collagen deposition in the large intestine of Nrf2 transcriptional knockout mice ($tNrf2^{-/-}$). The studies were performed on six-month-old female mice $Nrf2^{+/+}$ and $tNrf2^{-/-}$ randomly assigned to Avertin (250 mg/kg b.w. single *i.p.* injection) or vehicle group. We observed a 2-fold increase in anaesthesia time and longer recovery time ($p = 0.015$) in $tNrf2^{-/-}$ in comparison to $Nrf2^{+/+}$. However, no hepato- or nephrotoxicity was detected. Interestingly, we found severe changes in colon morphology of untreated $tNrf2^{-/-}$ mice associated with colon shortening ($p = 0.02$) and thickening ($p = 0.015$). Avertin treatment caused colon damage manifested with epithelial layer damage and goblet depletion in $Nrf2^{+/+}$ mice but not in $tNrf2^{-/-}$ individuals. Additionally, Avertin did not induce oxidative stress in colon tissue, but it increased leukocyte infiltration in $Nrf2^{+/+}$ mice ($p = 0.02$). Immunofluorescent staining also revealed enhanced deposition of collagen I and collagen III in the colon of untreated $tNrf2^{-/-}$ mice. Avertin contributed to increased deposition of collagen I in $Nrf2^{+/+}$ mice but reduced deposition of collagen I and III in $tNrf2^{-/-}$ individuals. In conclusion, $tNrf2^{-/-}$ respond to Avertin with prolonged anaesthesia that is not associated with acute toxicity, inflammatory reaction or enhanced oxidative stress. Avertin does not impair intestine morphology in $tNrf2^{-/-}$ mice but can normalise the enhanced fibrosis.

## 1. Introduction

2,2,2-tribromoethanol (TBE; Avertin) has been used since 1926 as an anaesthetic for dogs, cats, mice and rats [1,2]. It was usually injected intraperitoneally in a single dose of 240 − 250 mg/kg and provided short-term anaesthesia [3]. After administration, Avertin is metabolised in the liver and excreted with the urine probably in a similar way to other alcoholic xenobiotics [4]. However, its metabolism has not yet been described in detail.

Although its use is considered safe in most common mouse strains [5], studies are indicating its irritant and proinflammatory properties. For instance, Thompson et al. [6] presented that even a single dose of Avertin may cause damage of Kupffer and endothelial cells in the liver and may increase the level of aminotransferases at 3 and 6 h after injection. Moreover, Avertin may irritate the intestine leading to fibrosis [7]. Nevertheless, it has been commonly used as a general anaesthetic, since it is easy to prepare and rapidly induces anaesthesia with fast recovery [8], along with low mortality and morbidity rate (< 1%) [9]. As Avertin is an alcoholic xenobiotic, it is of particular importance to verify its effectiveness and safety in individuals with impaired detoxification and antioxidative reaction, such as Nrf2 (nuclear factor-erythroid 2 related factor 2)-defective mice.

Nrf2 is a transcription factor, which plays an essential role in the regulation of detoxification, antioxidative, and anti-inflammatory responses [10,11] by binding to the antioxidant response elements (ARE) in the promoters of target genes [12]. The Nrf2 transcriptional knockout ($tNrf2^{-/-}$) mice are viable and fertile but show increased sensitivity to different xenobiotics including benzo(a)pyrene, butylated hydroxytoluene, acetaminophen, and dextran sulphate sodium [reviewed in [13,14]]. Interestingly, although Nrf2 induces expression of

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antioxidative genes, the vascular system of tNrf2$^{-/-}$ mice does not show any massive oxidative damage in steady-state conditions, despite increased production of reactive oxygen species. Instead, the lack of Nrf2 transcriptional activity drives the onset of premature senescence [15] and protein aggregation [16] through non-canonical, Keap1 dependent mechanisms [17]. Moreover, inhibition of Nrf2-regulated genes may have a protective role against colon diseases [18–20]. It was also shown that Nrf2 plays an essential role in colon fibrosis observed in patients with ulcerative colitis and Crohn's disease where inflammation, thickening of *muscularis mucosae* and collagen accumulation are observed what may cause colon shortening [21].

To date, there is no information on how Avertin acts in mice with impaired Nrf2 signalling. Therefore, the aim of this study was to analyse the influence of a single dose of Avertin on anaesthesia duration time, inflammatory response, oxidative stress and collagen deposition in the large intestine of Nrf2 transcriptional knockout mice.

## 2. Material and methods

### 2.1. Animals and housing

Experiments were performed on C57/BL6 mice with the normal level of Nrf2 (Nrf2$^{+/+}$), and on B6.129 × 1-Nfe2l2$^{tm1Ywk}$ mice lacking transcriptionally active Nrf2 (tNrf2$^{-/-}$). Mice were generated by Prof. Masayuki Yamamoto [12] and kindly provided by Prof. Antonio Cuadrado (Universidad Autonoma de Madrid, Spain). In tNrf2$^{+/+}$ mice, the DNA binding domain of Nrf2 was replaced by *LacZ* gene, to generate a fusion protein N-terminal Nrf2-β-galactosidase [22,23].

The animals were maintained in the SPF (specific pathogen-free) animal facility at the Faculty of Biochemistry, Biophysics and Biotechnology, under a constant 12 h dark/light cycle at the environmental temperature of 22 ± 2 °C and were provided with standard laboratory pellet diet and water *ad libitum*. For experiments, 6-month-old female mice with verified genotype by PCR method were used.

The study was carried out in accordance with the European Communities Council Directive (2010/663/UE) and with the national recommendations. The experimental protocol was approved by the Second Local Ethics Committee for Animal Research in Krakow (No. 77/2018).

### 2.2. Avertin efficacy and toxicity

Nrf2$^{+/+}$ and tNrf2$^{-/-}$ female mice were randomly assigned to one of two groups to receive a single *i.p.* injection of Avertin (250 mg/kg b.w.; 1.2 % solution, 0.5 mL) or vehicle (0.5 mL), mentioned as control. Avertin treated mice contained 6 animals and control mice contained 5 animals. Avertin was prepared as recommended at the Baylor College of Medicine [24]. Briefly, a 1.6-g/mL stock solution was prepared by adding 0.5 mL tert-amyl alcohol (Sigma–Aldrich) to 0.25 g 2,2,2-tribromoethanol (99 %; Sigma-Aldrich). A 20-mg/mL working solution was prepared by adding 0.5 mL of the tribromoethanol stock solution to 39.5 mL sterile saline (Polpharma); the working solution was filtered through a 0.2-mm syringe filter (Fisher Scientific). The stock solution was made 1 day before injection and allowed to stir overnight at room temperature; the working solution was made immediately prior to injection. Vehicle was prepared as above without the addition of 2,2,2-tribromoethanol.

After Avertin injection mice were placed on a digital thermoregulated heating pad (37 °C, Techniplast) with eye lubricant for protection, and the duration of anaesthesia, and recovery time were monitored. The anaesthetic duration was defined as the time between the loss and return of the pedal reflex [minutes]. Presence of the pedal reflex was defined as the withdrawal of the limb after pinching with tweezers. Recovery time [minutes] was defined as mouse ability to maintain an upright posture and walking normally around the cage.

Next, 3 h and 6 h after injection the venous blood was collected to serum separator tubes to assess the levels of glucose and selected markers of liver damage (alkaline phosphatase (ALP) and alanine aminotransferase (ALT)) and kidney damage (creatinine, urea nitrogen, and total protein). Biochemical analysis was made in 100 µL of serum using SpotChem EZ Analyzer and ready-to-use stripes (Panel V; Woodley equipment).

### 2.3. Colon harvesting

Twenty-eight days after treatment mice were sacrificed by exposure to carbon dioxide inhalation, followed by cervical dislocation. Next, large intestine was dissected and subjected to macroscopic scoring of colon length and wall thickness, followed by tissue preservation for myeloperoxidase (MPO) activity assay, malondialdehyde (MDA) assay, histological staining, and gene expression analysis.

### 2.4. Histological stainings

After macroscopic evaluation, segments of the distal colon were stapled flat, mucosal side up, onto cardboard and fixed in 10 % neutral-buffered formalin for 24 h at 4 °C. Samples were then dehydrated, embedded in paraffin, sectioned at 5 µm (microtome Thermo Fisher Scientific), and mounted onto slides. Next, specimens were deparaffinised and stained using haematoxylin and eosin (H&E; for general morphology) or Trichrome staining (for collagen detection) using tissue strainer (Thermo Fisher Scientific) and commercially available reagents and kits (Sigma-Aldrich). Samples were analysed under a light microscope (Nikon, Eclipse Ti-S). Each time 3 separate tissue scraps from the same sample were analysed.

### 2.5. Microscopic scoring

Microscopic scoring was performed as previously described [25]. Sections stained with H&E were examined using a Nikon Eclipse Ti-S microscope. Photographs were taken and analysed using Image J software (Wayne-Rasband (NIH)). Microscopic total damage score was determined in blind manner based on the presence (score = 1) or absence (score = 0) of goblet cell depletion, the presence (score = 1) or absence (score = 0) of crypt abscesses, the destruction of mucosal architecture (normal = 1, moderate = 2, extensive = 3), the extent of muscle thickening (normal = 1, moderate = 2, extensive = 3), and the presence and degree of cellular infiltration (normal = 1, moderate = 2, transmural = 3).

Change in collagen content (Trichrome staining) was assessed basing on the intensity of collagen (blue colour) staining in the intestinal crypts, *muscularis mucosa* and *submucosa* using the following scale: 1 = small, 2 = medium, 3 = excessive collagen intensity staining. The total sum of points indicated the final collagen content in the sample. Each time 3 tissue scraps and 4 fields were counted. The analysis was blinded and performed independently by three researchers.

### 2.6. Immunofluorescence

Paraffinized samples were cut to 5 µm slices using a microtome (Thermo Fisher Scientific), deparaffinised and boiled for 15 min in a citric acid buffer (pH 6.0) to activate antigen, permeabilised in 0.01 % Triton X-100 for 2 min, washed and incubated with 0.25 % glycine in PBS for 30 min. Next, tissue scraps were blocked for 1 h in 3% goat serum (GS) at room temperature. After washing in PBS, samples were incubated overnight (4 °C) with rat anti-mouse CD45 polyclonal antibodies (dilution 1:50, Invitrogen) or rabbit anti-mouse collagen type I or type III antibodies (1:250, Abcam) diluted in 1% GS. At the next day, samples were washed and incubated with anti-rat or anti-rabbit antibody conjugated with Alexa Fluor 488 (dilution 1:1000; IgG H + L, Life Technologies) for 1 h at room temperature. Nuclei were counterstained with Hoechst 33342 (dilution 1:10,000). Samples were analysed under

a fluorescent microscope (Nikon, Eclipse Ti-S).

### 2.7. MPO assay

The myeloperoxidase (MPO) activity assay, which is an indicator of granulocyte infiltration [26], was performed as previously described by Salaga et al. [27]. Briefly, 1 cm segments of colon were weighed and homogenized in hexadecyltrimethylammonium bromide buffer (0.5 % in 50 mM potassium phosphate buffer, pH 6.0; 1:20 w/v). Next, the homogenate was centrifuged (15 min, 13,200 *g*, 4 °C) and 200 µl of 50 mM potassium phosphate buffer (pH 6.0), containing 0.167 mg/mL of O-dianisidine hydrochloride and 0.05 µl of 1% hydrogen peroxide was added to 7 µl of supernatant. Absorbance was measured at 450 nm (iMARK Microplate Reader; Biorad). MPO was expressed in milliunits per gram of wet tissue, 1 unit being the quantity of enzyme able to convert 1 µmol of hydrogen peroxide to water in 1 min at room temperature. Units of MPO activity per 1 min were calculated from a standard curve using purified peroxidase enzyme.

### 2.8. MDA assay

5 mg of large intestine tissue was homogenised in 200 µL of lysis buffer (w/v) containing 20 % (w/v) trichloroacetic acid and 0.8 % (w/v) thiobarbituric acid (Sigma-Aldrich). Next, samples were boiled for 30 min and centrifuged (300*g*, 10 min). The absorbance of the supernatant was measured at 535 nm with the correction wavelength of 600 nm for the nonspecific turbidity (Infinite 200 Pro, Tecan). The amount of MDA was calculated using the extinction coefficient of MDA-TBA complex, which is $1.56 \times 10^5\ M^{-1}\ cm^{-1}$, and the results were expressed as mM MDA/mg tissue.

### 2.9. Analysis of gene expression

RNA from 30 mg of large intestine tissue was extracted with RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesised using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). RT-qPCR was conducted on Step-One Plus Real-Time PCR Systems using a Power SYBR® Green PCR Master Mix according to the manufacturer's instructions (Thermo Fisher Scientific). Primer sequences and their efficiency are gathered in Table 1. Eukaryotic mouse translation elongation factor 2 (*Eef-2*) was used as a reference gene. The results were calculated using the relative expression method.

### 2.10. Statistical analysis

Data are presented as mean ± S.E.M. with individual values. Depending on the normality of distribution, the comparisons between groups were performed using the Student's *t*-test or Mann-Whitney test for two-group comparisons. Multiple comparisons were made using two-way or three-way analysis of variance (ANOVA), followed by the Dunn's post-hoc test (GraphPad Prism software).

## 3. Results

### 3.1. Avertin causes prolonged anaesthesia and delayed recovery in mice with transcriptionally inactive Nrf2

Mice with transcriptionally inactive Nrf2 ($tNrf2^{-/-}$) responded differently to Avertin-induced anaesthesia than their wild type ($Nrf2^{+/+}$) counterparts. We observed a 2-fold increase in anaesthesia time in $tNrf2^{-/-}$ in comparison to $Nrf2^{+/+}$ counterparts (21 ± 3 min *vs* 9 ± 1 min; $p = 0.02$; Fig. 1A). Moreover, $tNrf2^{-/-}$ mice had prolonged recovery time (72 ± 5 *vs* 53 ± 5 min; $p = 0.015$; Fig. 1B). These results might suggest that animals with impaired transcriptional activity of Nrf2 may metabolise Avertin more slowly than mice with the normal transcriptional activity of Nrf2.

### 3.2. A single dose of Avertin provokes no hepato- and nephrotoxicity in $tNrf2^{-/-}$ mice

To verify the toxicity of a single dose of Avertin, we analysed changes in selected biochemical parameters, associated with liver and kidney function - main metabolising organs of Avertin - in the blood at 3 h and 6 h post-treatment (Fig. 2). We found that a single dose of Avertin in wild type mice caused a significant increase in ALT at both time points ($p = 0.04$ for 3 h and $p = 0.02$ for 6 h; Fig. 2A), however, a decrease in ALP after 3 h ($p = 0.04$, Fig. 2B) compared to the untreated control. Of note, the level of ALT was at the lower range of grade 1 hepatotoxicity score, which is 51–125 IU/L [28]. No significant changes in ALT and ALP were observed in $tNrf2^{-/-}$ mice treated with Avertin (Fig. 2A,B). On the other hand, in tNrf2 mice serum there was a decrease in creatinine ($p = 0.03$; Fig. 2C), total protein level ($p = 0.02$) but not urea nitrogen (Fig. 2D) after 3 h compared to the control. A similar trend, although less pronounced, was also observed in the $Nrf2^{+/+}$. No significant changes in plasma glucose were detected in both genotypes (Fig. 2E,F). Therefore, a single dose of Avertin provokes some changes in liver-damage markers in $NRf2^{+/+}$ mice and kidney-damage markers in $Nrf2^{-/-}$ animals. However, Avertin is not hepatotoxic and does not change ASP and ALT levels in $tNrf2^{-/-}$ mice, despite a significant influence on anaesthesia duration time.

### 3.3. A single dose of Avertin impairs colon morphology in $Nrf2^{+/+}$ but not in $tNrf2^{-/-}$ mice

Different response to anaesthetic treatment may result from altered colon absorption. Thus we inspected it further. We observed significant changes in colon morphology. Macroscopic analysis showed that the colon was shorter ($p = 0.02$, Fig. 3A) and tended to be thicker ($p = 0.15$, Fig. 3B) in the untreated $tNrf2^{-/-}$ than in $Nrf2^{+/+}$ mice. Which was also visible at microscopic level on histological slides as thickening of the muscular layer (marked with asterisk on Fig. 3C). Those changes

**Table 1**
The sequence of primers used in the study.

| Gene | Primer sequence 5′-3′ | Primer efficiency [%] |
|---|---|---|
| Mouse *Col1α1* | F: 5′- ACTACCGGGCCGATGATGCTAACG -3′ R: 5′- CGATCCAGTACTCTCCGCTCTTCC -3′ | 113.7 |
| Mouse *Col1α2* | F: 5′- GCCACCATTGATAGTCTCTCC -3′ R: 5′- CACCCCAGCGAAGAACTCATA -3′ | 115.8 |
| Mouse *Col3α1* | F: 5′- ATCTATGAATGGTGGTTTTCAGTT -3′ R: 5′- TTTTGCAGTGGTATGTAATGTTCT -3′ | 108.2 |
| Mouse *Col4α1* | F: 5′- CAGATTCCGCAGTGCCCTA -3′R: 5′- GGAATAGCCGATCCACAGTGAG -3′ | 104.4 |
| Mouse *TNF-α* | F: 5′- GACCCTCACACTCAGATCATCTTCT-3′ R: 5′- CCACTTGGTGGTTTGCTACGA -3′ | 118.2 |
| Mouse *IL-4* | F: 5′- CTGGTGTGTGACGTTCCCATTA -3′ R: 5′- CCGACAGCACGAGGCTTT -3′ | 97.6 |
| Mouse *IL-1β* | F: 5′- CTGGTGTGTGACGTTCCCATTA-3′ R: 5′- CCGACAGCACGAGGCTTT -3′ | 114.5 |
| Mouse *IL-6* | F: 5′-AAAGAGTTGTGCAATGCAATGGCAATTCT-3′ R: 5′- AAGTGCATCATCGTTGTTCATACA -3′ | 98.2 |
| Mouse *MCP-1* | F: 5′- GGCTCAGCCAGATGCAGTTAA -3′ R: 5′- CCTACTCATTGGGACTCATCTTCT -3′ | 107.2 |
| Mouse *Ef-2* | F: 5′- GACATCACCAAGGGTGTGCA -3′ R: 5′- TCAGCACACTGGCATAGACC -3′ | 92.4 |

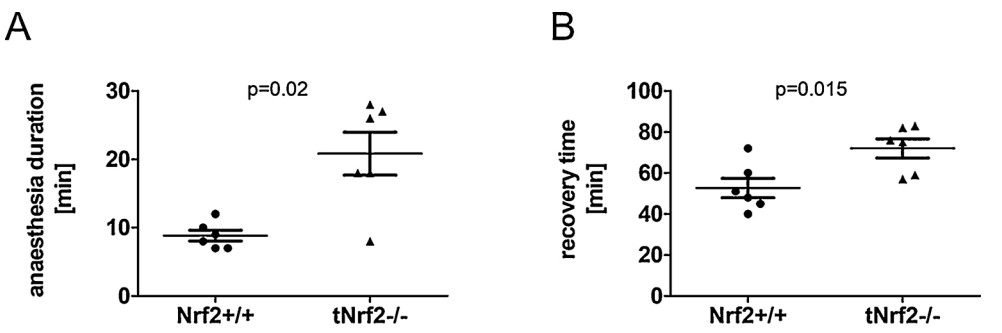


**Fig. 1.** Avertin causes prolonged anaesthesia and delayed recovery in mice with transcriptionally inactive Nrf2. Duration of (**A**) anaesthesia, and (**B**) recovery in 6-month-old female Nrf2$^{+/+}$ and tNrf2$^{-/-}$ mice divided randomly into control (n = 5) and Avertin (n = 6) groups. Mice were treated intraperitoneally with a single dose of Avertin or vehicle. Values are expressed as mean ± S.E.M. Two-tailed Student's *t*-test.

were not influenced by different body weight in both genotypes (24.98 ± 0.34 g for Nrf2$^{+/+}$ *vs* 23.26 ± 0.76 g for tNrf2$^{-/-}$; p = 0.11). Thus, lack of transcriptionally active Nrf2 worsens colon morphology in adult female mice.

Of note, as the experiments were performed on 6 month-old mice, some age-related changes in the colon structure were noticed in control Nrf2$^{+/+}$ animals and were reflected in higher microscopic scoring than expected (Fig. 3D).

Looking further, and given the fact that Avertin may lead to intestinal fibrosis [7], we checked the long term effect of a single dose of Avertin on the colon morphology. Four weeks after administration, Avertin did not significantly influence the colon length or colon thickness in the wild type mice, but it tended to attenuate the difference between the length of colons in tNrf2$^{-/-}$ individuals (p = 0.057; Fig. 3A). However, histological analysis indicated that Avertin impaired colon morphology in Nrf2$^{+/+}$ mice manifested as epithelial layer damage and goblet depletion (marked with arrows on Fig. 3C). All those differences were reflected in the higher macroscopic score in Avertin treated Nrf2$^{+/+}$ mice compared to the control (p = 0.015; Fig. 3D). On the contrary, Avertin-treated tNrf2$^{-/-}$ mice underwent positive changes in colon morphology represented as a thinner muscular layer (Fig. 3C and Suppl. 1) and lower microscopic score compared to Avertin-treated Nrf2$^{+/+}$ (p = 0.03; Fig. 3D). Thus, Avertin contributes to colon damage in Nrf2$^{+/+}$ mice but improves the disturbed colon morphology in tNrf2$^{-/-}$ individuals.

### *3.4. Colon morphological changes in tNrf2$^{-/-}$ mice treated with Avertin are accompanied by altered expression of collagens but not with oxidative stress or inflammation*

To shed some light on possible causes of changes in colon morphology in the untreated and Avertin-treated tNrf2$^{-/-}$ mice, we evaluated oxidative stress, inflammation and expression of collagens in the colon wall. The results showed that the untreated tNrf2$^{-/-}$ mice have a similar level of MPO activity and lipid peroxidation in the colon compared to the untreated wildtype control and that Avertin did not influence these parameters in any of the genotypes. Therefore, we suspect that excessive oxidative stress is not the reason for colon damage in tNrf2$^{-/-}$ mice and Nrf2$^{+/+}$ mice treated with Avertin (Fig. 4).

Further, there was no difference between the untreated animals of both genotypes in the inflammatory response, as leukocyte infiltration (CD45$^{+}$ cells) and levels of proinflammatory cytokines (TNFα, IL-6, IL-1β, MCP-1) were similar (Fig. 5A-C). Treatment with Avertin increased leukocyte infiltration (p = 0.02; Fig. 5A,B) and TNFα mRNA level (p = 0.009; Fig. 5C) in the wild type mice. Surprisingly, the tNrf2$^{-/-}$ mice were resistant to Avertin-mediated proinflammatory changes.

Given the fact that abnormal collagen production and deposition is conducive for many colon-related disorders and is directly associated with inflammatory response, we analysed the expression of collagens within the colon tissue. First, we inspected total collagen content by Trichrome staining (Fig. 6A,B). It revealed that untreated tNrf2$^{-/-}$ mice have higher collagen content comparing to their wildtype counterparts (p = 0.04; Fig. 6A,B). Moreover, while Nrf2$^{+/+}$ mice treated with Avertin showed a tendency to increased expression of *Col1a1* mRNA, a similar trend was not observed in tNrf2$^{-/-}$ mice. Instead,

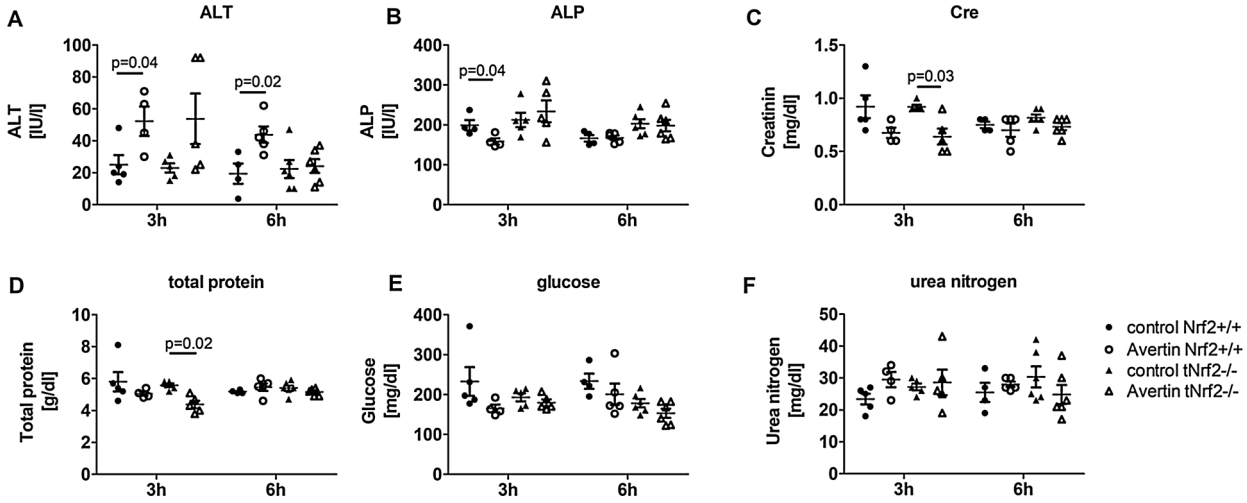


**Fig. 2.** A single dose of Avertin provokes no hepato- and nephrotoxicity in tNrf2$^{-/-}$ mice. Serum concentration of liver-damage markers (**A**) alanine aminotransferase (ALT); (**B**) alkaline phosphatase (ALP); kidney-damage markers (**C**) creatinin (Cre); (**D**) total protein; (*E*) urea nitrogen, and (**F**) glucose in 6 month old female Nrf2$^{+/+}$ and tNrf2$^{-/-}$ mice treated intra peritoneally with a single dose of Avertin or vehicle. Mice were divided randomly into control (n = 5) and Avertin (n = 6) groups. Data are presented as mean ± S.E.M. Three-way ANOVA with Dunn's post-hoc test.

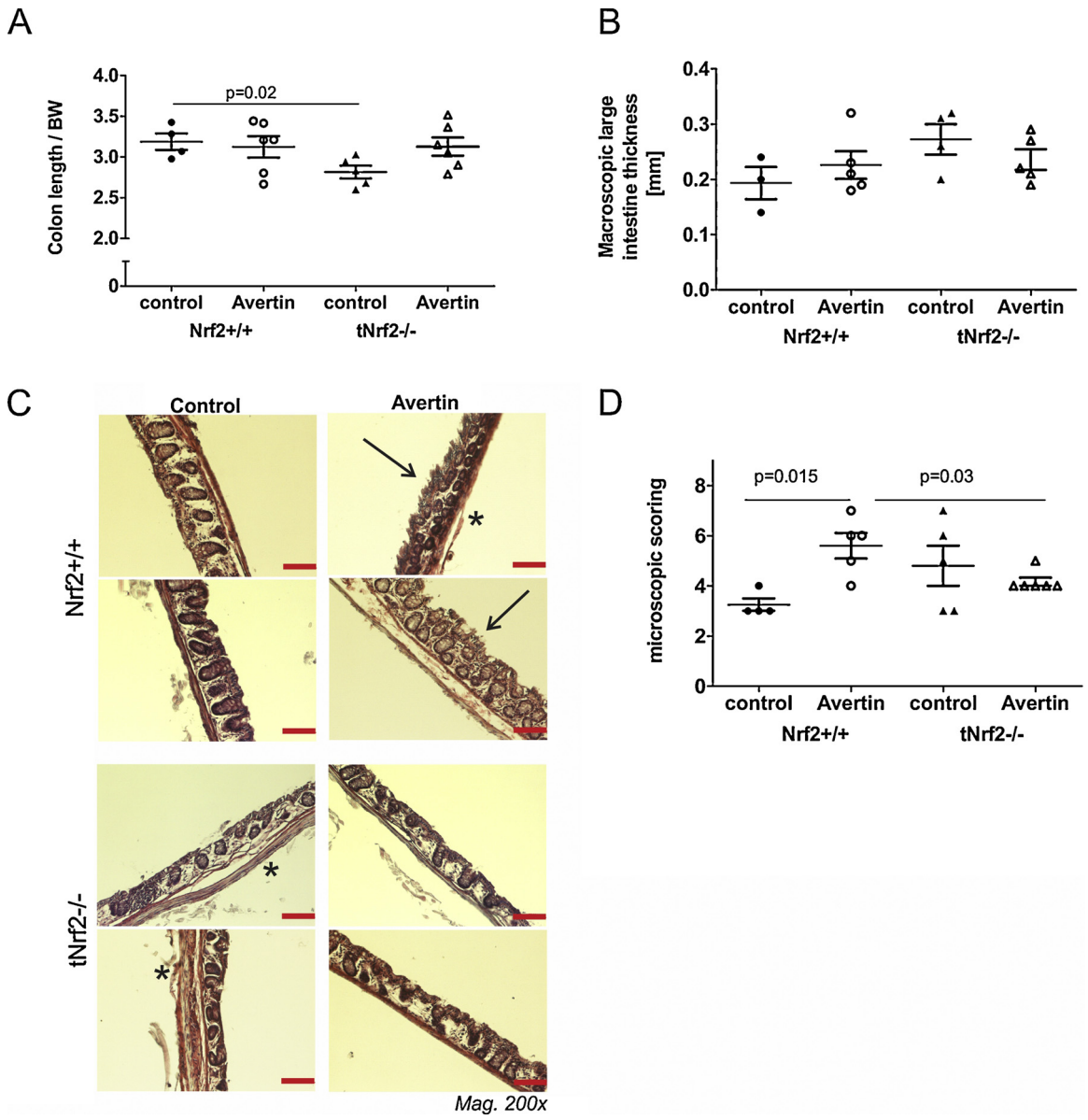


**Fig. 3.** A single dose of Avertin impairs colon morphology in Nrf2$^{+/+}$ but not in tNrf2$^{-/-}$ mice. The change in (**A**) colon length to body weight (BW); (**B**) macroscopic large intestine thickness; (**C**) representative images of microscopic changes in colon morphology (H&E staining at magnification 200*x*; arrow - goblet cells depletion; asterisk - *muscularis mucosae* thickening); and (**D**) microscopic scoring (based on histological slides) of the colon in mice 28-days after a single dose of Avertin (n = 6) or vehicle (n = 5). Data in panels A, B and D are presented as mean ± S.E.M. Two-way ANOVA with Dunn's post-hoc test.

there was a decrease in the expression of *Col3a1* (p = 0.02) and a tendency for increased expression of *Col4a1* transcript (Fig. 6C). Immunofluorescent staining revealed enhanced deposition of collagen I and collagen III in the colon of untreated tNrf2$^{-/-}$ mice and mainly collagen I in the Avertin treated Nrf2$^{+/+}$ mice (Fig. 6D). Accordingly, analysis of mRNA expression, the immunostaining showed that a single dose of Avertin causes increased deposition of collagen I in Nrf2$^{+/+}$ mice but reduces deposition of collagen I and III in tNrf2$^{-/-}$ individuals (Fig. 6D,E).

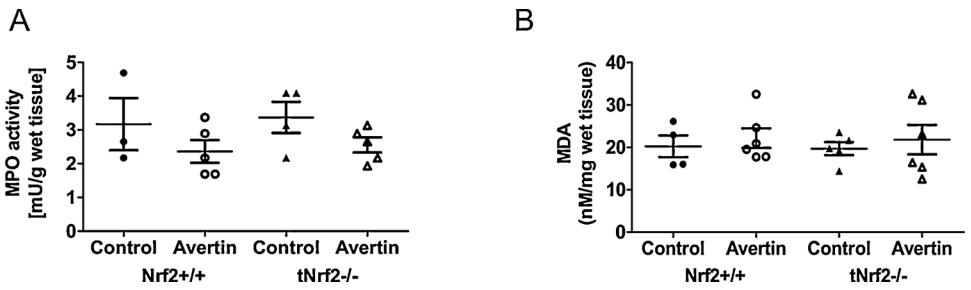


**Fig. 4.** Avertin treatment has no influence on colon oxidative stress. The changes in (**A**) MPO activity and (**B**) lipid peroxidation (MDA) in mice 28-days after a single dose of Avertin or vehicle. Mice were divided randomly into control (n = 5) and Avertin (n = 6) groups. Data are presented as mean ± S.E.M. Two-way ANOVA with Dunn's post-hoc test.

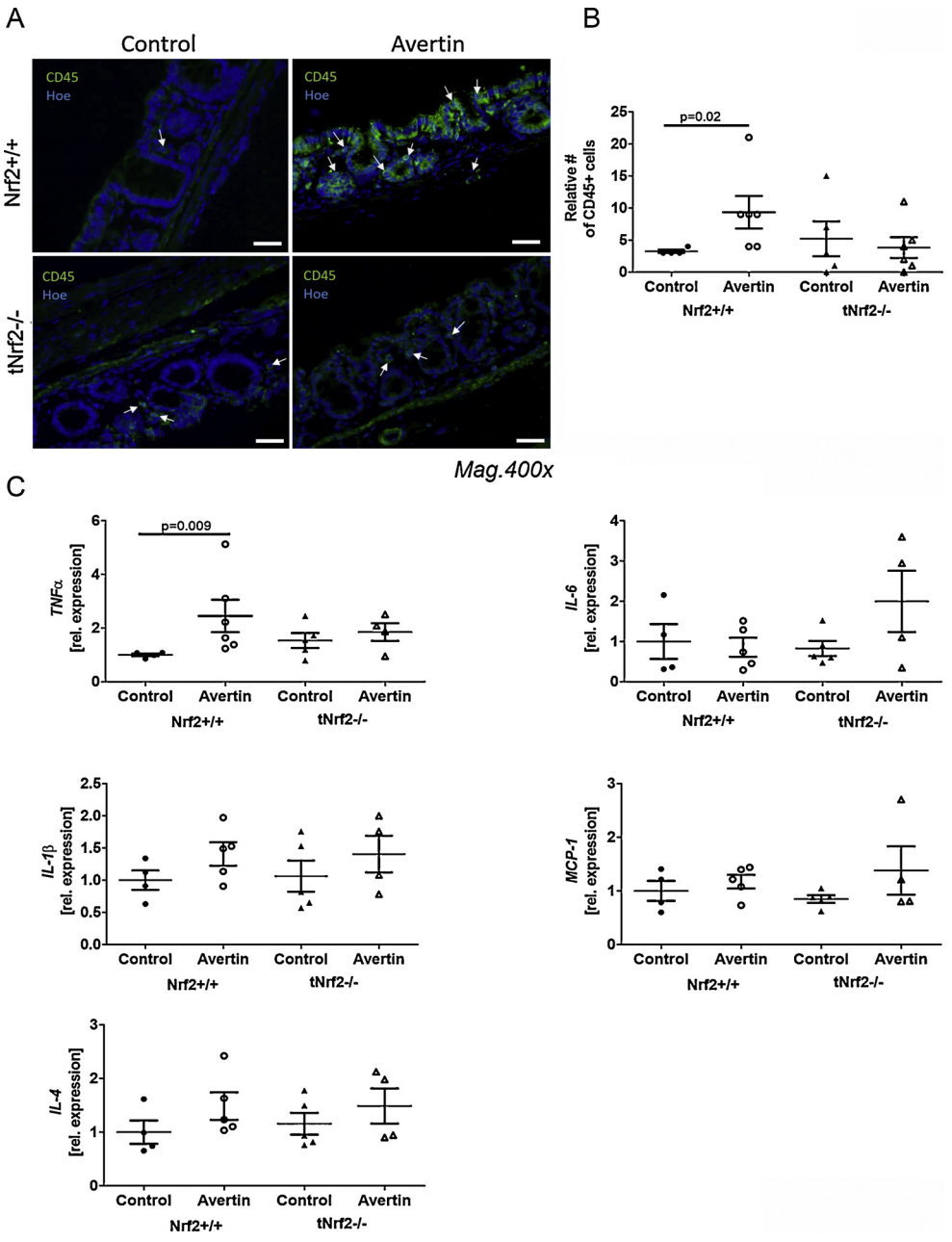


**Fig. 5.** Colon morphological changes in tNrf2$^{-/-}$ mice are not associated with inflammatory response. (**A**) Representative images of immunofluorescent staining of CD45 cells within the colon (magnification 400 × . CD45 - green, nuclei - blue, arrows indicate CD45$^{+}$ cells) with (**B**) relative number of CD45$^{+}$ cells infiltrating colon tissue; (**C**) relative expression of *TNFα*, *IL-6*, *IL-1β*, *MCP-1,* and *IL-4*. *Eef2* was used as a reference gene. Mice were sacrificed 28-days after a single administration of Avertin (n = 6) or vehicle (n = 5). Data in panels B and C are presented as mean ± S.E.M. Two-way ANOVA with Dunn's post-hoc test. (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).

## 4. Discussion

We show in this study that Avertin is non-toxic, yet it causes prolonged anaesthesia in mice with transcriptionally inactive Nrf2. We also found that these mice have changed morphology and collagen distribution in the large intestine when compared to the wild type animals. Treatment with Avertin did not further impair those changes but instead tended to improve them. On the other hand, even a single dose of Avertin may adversely affect the morphology of intestine in the wild type animals. The above should be taken into account when planning anaesthesia.

It is known that response to anaesthetic strongly depends on metabolism rate and body antioxidant ability. A recent study showed that exposure of rats to injected (propofol) or inhaled (isoflurane) anaesthetics induced enhanced glutathione (GSH) production, which results from the Nrf2-dependent expression of the excitatory amino acid carrier 1 (EAAC1) in the hippocampus [29]. It is known that different anaesthetics may activate Nrf2 *via* phosphoinositide-3-kinase and protein kinase B (PI3K/Akt), contributing to an enhancement of antioxidant response [30,31]. Therefore, Nrf2 activation likely acts as a defence mechanism against oxidative stress during anaesthesia. However, tNrf2$^{-/-}$ animals did not show signs of significant oxidative stress even after treatment with Avertin, which supports our earlier observations [15,32]. Hence, prolonged anaesthetic time does not seem to be oxidative stress-dependent, but rather might be related to impaired detoxification of xenobiotics [11,12].

Moreover, despite prolonged anaesthesia, no changes in ASP, ALT or creatinine was observed in the blood of tNrf2$^{-/-}$ mice, revealing no acute hepato- or nephrotoxicity of Avertin. This observation was surprising, as activation of Nrf2 reduced or prevented the xenobiotics-induced toxicity in many experimental settings [reviewed in [33]]. Interestingly, we found that the colon structure in tNrf2$^{-/-}$ mice resembled the one observed during dextran sulphate sodium (DSS)-induced colitis [34]. Thus, we suppose that the prolonged anaesthesia in case of tNrf2$^{-/-}$ mice might be associated with impaired Avertin absorption within the intestine.

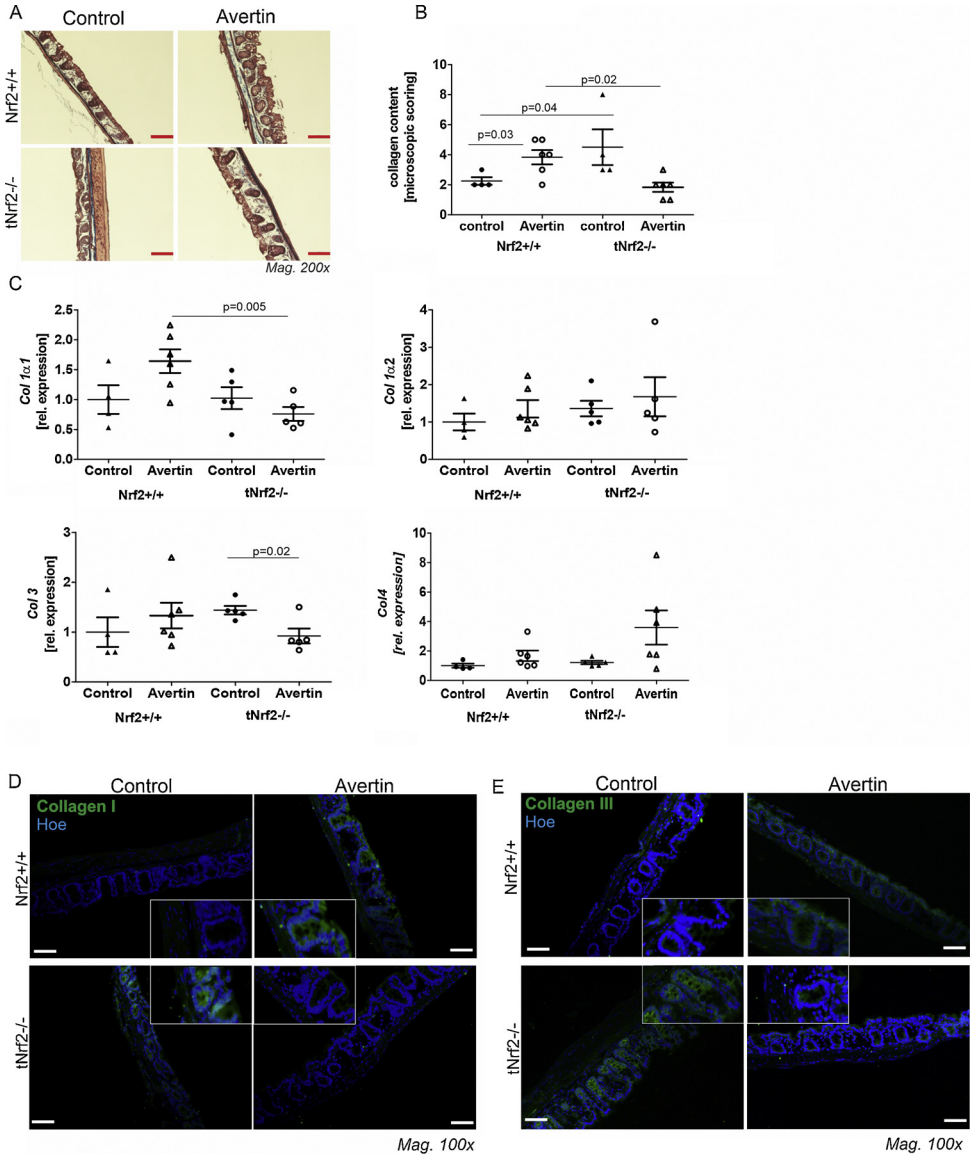


**Fig. 6.** Colon morphological changes in tNrf2$^{-/-}$ mice treated with Avertin are accompanied by altered expression of collagens. (**A**) Representative images of total collagen content (blue; magnification 200*x*) with (**B**) microscopic scoring of total collagen; (**C**) relative expression of *Col1α1*, *Col1α2*, *Col3*, and *Col4*. *Eef2* was used as a reference gene; and (**D**) representative immunofluorescent staining of collagen type I and III within the colon (magnification 100*x*. Collagen - green, nuclei – blue). Mice were sacrificed 28-days after a single administration of Avertin (n = 6) or vehicle (n = 5). Data in panels B and C are presented as mean ± S.E.M. Two-way ANOVA with Dunn's post-hoc test. (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).

We also found that, differently than in the wild type animals, a single dose of Avertin can improve colon morphology in the tNrf2$^{-/-}$. One could expect that such improvement should be related to decreased inflammation or oxidative stress. Nrf2 is a key transcription factor which influences the expression of antioxidant and anti-inflammatory genes [35]. It was previously reported that Nrf2-deficient mice had increased level of some proinflammatory cytokines, which could predispose them to DSS-induced colitis [36]. On the other hand, it was shown that prolonged activation of Nrf2 triggers inflammation and increases the risk of colon cancer [37]. However, in our experimental settings, the colon lengthening in tNrf2$^{-/-}$ mice treated with Avertin was independent of oxidative stress or inflammation. Of note, Nrf2 plays a role in the release of calcium ions and limits excessive stimulation of the sympathetic system, which influence contractility of colon tissue [38]. In accordance, Nrf2-deficient mice show a higher susceptibility of the colon to acetylcholine [39].

Another cause of improvement of colon morphology in tNrf2$^{-/-}$ mice after Avertin could be related to changes in the production of extracellular matrix (ECM) proteins, mainly collagens. In our study, the lack of transcriptionally active Nrf2 resulted in a significant deposition of collagen, particularly type III, mostly within *lamina propria*. Inflammatory colon diseases are associated with tissue fibrosis [40]. It was previously shown that Nrf2-deficient mice have higher expression of collagen type I, III and fibronectin in the skin [41]. Additionally, impaired tissue regeneration was observed in those mice [42]. In accordance, Nrf2 activation protects against fibrosis in lungs [43], whereas protracted activation of Nrf2 results in overexpression of P450 cytochromes, inflammation, fibrosis and eventually cancer formation [44]. What is more, Nrf2 activation during xenobiotics metabolism was also associated with retardation of structural cytoskeletal and ECM proteins [45]. Therefore, Nrf2 activity can regulate the production of ECM proteins within large intestine tissue. Our results suggest that a single dose of Avertin administered to tNrf2$^{-/-}$ mice may reduce the collagen accumulation in the intestine. On the other hand, in the wild type animals, Avertin can induce colon fibrosis which may be associated with higher deposition of collagens. Nevertheless, further studies on the role of Nrf2 in xenobiotics absorption and metabolism should be addressed. Deepen examination of the influence of transcriptional activity of Nrf2 in the colon development as well as functionality, which may depend not only on sex but also on age could shed a light on this sparsely understood topic.

In conclusion, mice lacking transcriptionally active Nrf2 respond to Avertin with prolonged anaesthesia that is not associated with acute toxicity, inflammatory reaction or enhanced oxidative stress. Moreover,

Avertin does not impair intestine morphology in Nrf2$^{-/-}$ mice but can normalise the enhanced fibrosis.

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## Authors contribution

Participated in research design: APP, AK. Conducted experiments: APP, AK, EW, DK, KH. Performed data analysis: AK, APP. Wrote or contributed to the writing of the manuscript: APP, AK, AJ, JF.

## Declaration of Competing Interest

The author(s) declared no potential conflicts of interest concerning the research, authorship, and/or publication of this article.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2020.110317.

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