

Supplementary Materials

Article

Inflammation as a Possible Trigger for Mitoxantrone-induced Cardiotoxicity: an in vivo study in adult and infant mice

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Table S1. Scoring sheet for evaluation of the general welfare of mice during the experimental protocol.

	Clinical symptoms assessed	Score
Attitude/ Activity	Alert and active animal, normal posture and coat	0
	Some apathy, immobility, decrease grooming	1
	Apathy, chromodacrioreia, loss of grooming	2
	Immobility, isolation, abnormal posture, chromodacrioreia, piloerection	3
	Animal in decubitus, little reaction	4
Weight loss/ stool consistency/ Bleeding	Normal growth (infant)/ normal weight (adult), normal stool consistency and no bleeding	0
	Does not grow according to the curve (infant)/ maintains weight (adult), soft stool consistency and no bleeding	1
	Growth below the curve (infant)/ weight decrease ~ 5%/ 48h (adult); very soft	2

	stool; slight bleeding	
	Zero growth (infant)/ weight decrease ~ 10%/ 24h (adult), dehydration ~ 5%; fluid stools; slight bleeding	3
	Weight decrease (infant)/ weight decrease > 15%/ 24h (adult), dehydration ~ 8%; watery diarrhea; gross bleeding	4
Body condition and ascites	Normal condition and absence of ascites	0
	Presence of sizable abdominal enlargement or ascites; possessing a volume of ascites (g)/body weight (g) of 1-5%	1
	Presence of sizable abdominal enlargement or ascites; possessing a volume of ascites (g)/body weight (g) of > 5%	2
	Presence of sizable abdominal enlargement or ascites; possessing a volume of ascites (g)/body weight (g) of > 10 %	3
	Sizable abdominal enlargement or ascites with loss of a righting reflex; possessing a volume of ascites (g)/body weight (g) of > 15 %	4

Specific score: 3-4 in one specific parameter = urgent evaluation;

Total Score: 6-9 = daily evaluation in multiple occasions; 9-12 = sacrifice and sample collection.

Grimace Scale		Score
Orbital tightening	Closing of the eyelid (narrowing of the orbital area)	0
	A wrinkle may be visible around the eye	1
	As a guideline, any eye closure that reduces the eye size by more than half should be coded as a "2".	2
Nose bulge	Bulging on the bridge of the nose	0
	Vertical wrinkles on the side of the nose	1
	In frontal headshots, a bulge may be seen as a widening of the nose area.	2
Check bulge		0
	Bulging on the cheeks	1
		2
Ear position	Ears rotate outwards and/or back, away from the face	0
	Ears may fold to form a "pinhead" shape	1
	Space between the ears increases	2
Whisker change	Whiskers are either pulled against the cheek, or pulled forward to "stand on end"	0
	Whiskers may clump together	1
	Whiskers lose their natural "downward" curve	2

Legend: 0: Not present, normal; 1: Moderate; 2: Obvious discomfort/pain

Intraperitoneal administration		Score
Response	No vocalization or general discomfort (GD)/ no abdominal pain (AP)	0
	Does not vocalize, GD (transient immobility, local irritation); AP < 5 minutes	1
	Vocalize, evident GD; AP < 10 minutes complete/ reversed with analgesia	2
	Vocalize, evident GD; AP > 10 minutes complete/ reversed with analgesia	3

Figure S1

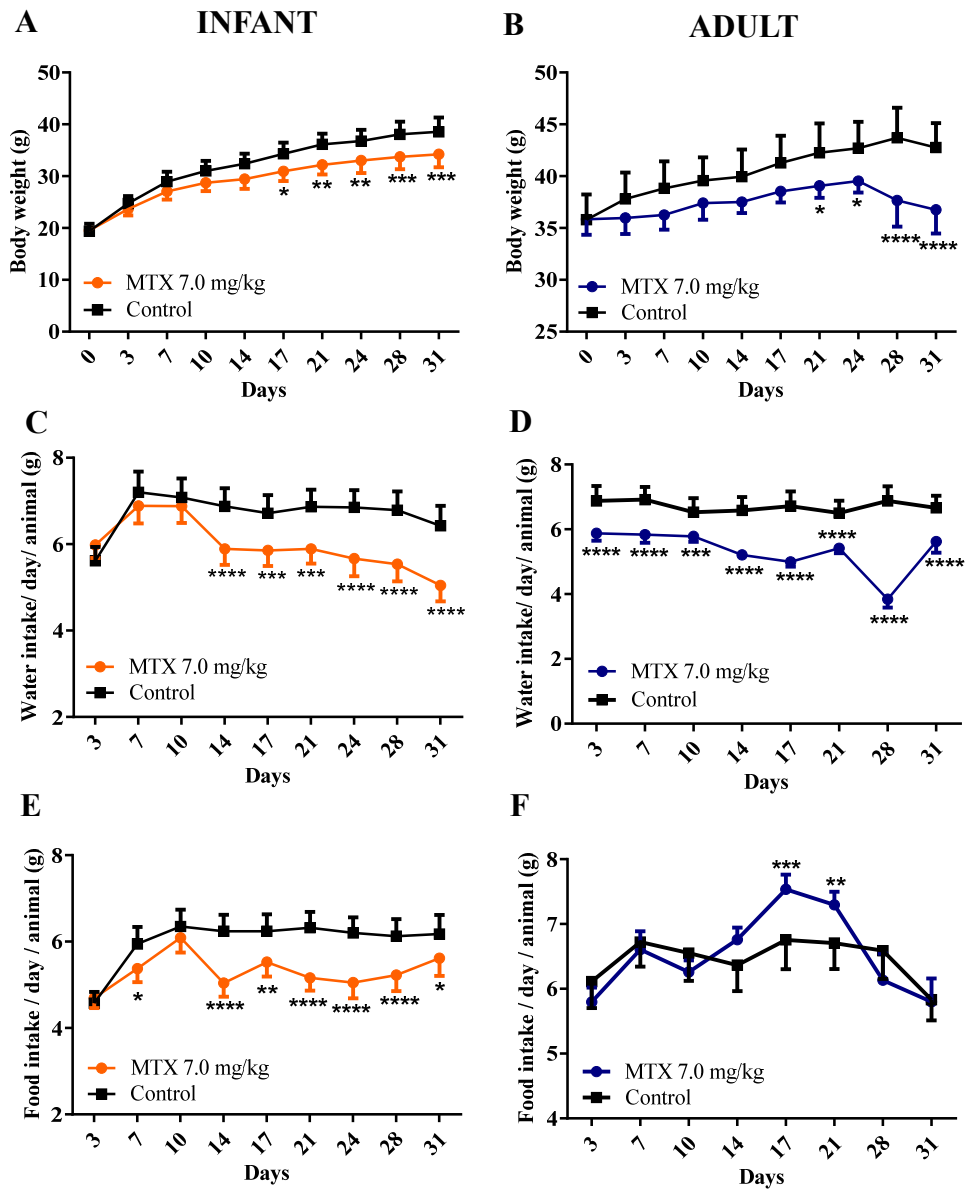


Figure S1. [A and B] Body weight in [A] infant and [B] adult mice exposed to a cumulative dose of 7.0 mg/kg MTX. [C and D] Water consumption of [C] infant and [D] adult mice exposed to a cumulative dose of 7.0 mg/kg MTX. [E and F] Food consumption of [E] infant and [F] adult mice exposed to a cumulative dose of 7.0 mg/kg MTX. Results are presented in grams (g) of food intake/day/weight of animal, mL of water intake/day/weight of animal or g of body weight, and as means $\pm$ standard deviation (SD), from 8 per group. Statistical comparisons were made using two-way ANOVA followed by the Sidak's *post hoc* test (* p < 0.05, ** p < 0.01, *** p < 0.001, and **** p < 0.0001, MTX 7.0 mg/kg vs. control).

Figure S2

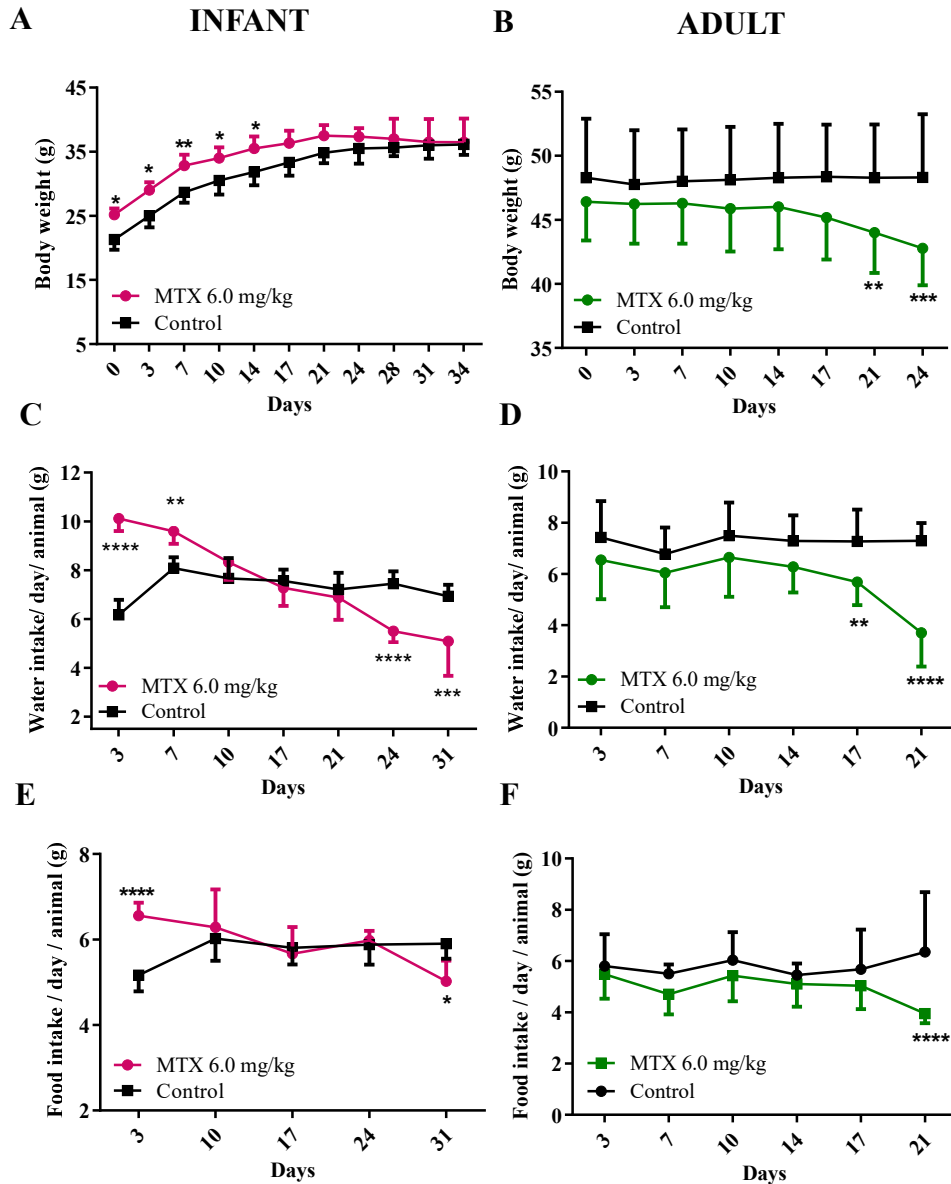


Figure S2. [A and B] Body weight in [A] infant and [B] adult mice exposed to a cumulative dose of 6.0 mg/kg MTX. [C and D] Water consumption of [C] infant and [D] adult mice exposed to a cumulative dose of 6.0 mg/kg MTX. [E and F] Food consumption of [E] infant and [F] adult mice exposed to a cumulative dose of 6.0 mg/kg MTX. Results are presented in grams (g) of food intake/day/weight of animal, mL of water intake/day/weight of animal or g of body weight, and as means $\pm$ SD, from 6 per infant group and 17 per adult group. Statistical comparisons were made using two-way ANOVA followed by the Sidak's *post hoc* test (* p < 0.05, ** p < 0.01, *** p < 0.001, and **** p < 0.0001, MTX 6.0 mg/kg vs. control).

Table S2. Plasma biomarkers and heart weight/body weight ratio after MTX (7.0 mg/kg cumulative dose).

	INFANT		ADULT	
Plasma				
	Control	MTX 7.0	Control	MTX 7.0 mg/kg
AST(U/L)	51.33 ± 8.31	70.20 ± 17.92	65.75 ± 21.79	87.13 ± 32.46
ALT (U/L)	37.00 ± 15.66	26.75 ± 3.30	39.63 ± 22.75	46.00 ± 37.04
AST/ALT ratio	1.69 ± 0.54	2.49 ± 0.40	1.80 ± 0.42	2.46 ± 0.91 (<i>p</i> = 0.06)
CK-MB (U/L)	73.67 ± 0.6.56	91.80 ± 14.96*	73.50 ± 33.12	99.25 ± 24.33
Total-CK (U/L)	45.14 ± 7.67	54.75 ± 4.03	78.38 ± 25.01	192.00 ± 141.93*
Heart				
Heart weight/ body weight ratio (%)	0.46 ± 0.04	0.45 ± 0.07	0.47 ± 0.05	0.42 ± 0.03*

Data of creatine-kinase - MB (CK-MB), total creatine-kinase (total CK), AST and ALT levels, in units per liter (U/L), are presented as means ± SD and were obtained from 4-8 animals of each group. Data of heart weight/body weight ratio, in percentage, are presented as means ± SD, and were obtained from seven (infant) and eight (adult) animals from each treatment group. Statistical comparisons were made using the t-test for CK-MB, total-CK and heart weight/body weight ratio in the adult group and the Mann-Whitney test for all other groups. **p* < 0.05, MTX 7.0 mg/kg vs. control.

Table S3. Plasma biomarkers and heart weight/body weight ratio after MTX (6.0 mg/kg cumulative dose).

INFANT			ADULT	
Plasma				
	Control	MTX 6.0 mg/kg	Control	MTX 6.0 mg/kg
AST(U/L)	37.67 ± 6.02	36.33 ± 20.62	58.00 ± 17.25	56.38 ± 20.63
ALT (U/L)	9.33 ± 4.27	34.52 ± 9.83**	25.60 ± 9.96	26.88 ± 8.52
AST/ALT ratio	4.81 ± 2.37	1.23 ± 0.23**	2.36 ± 0.66	2.27 ± 0.96
CK-MB (U/L)	-	-	77.22 ± 19.64	77.22 ± 16.82
Total-CK (U/L)	-	-	45.40 ± 29.77	58.00 ± 32.18
Heart				
Heart weight/ body weight ratio (%)	0.52 ± 0.04	0.47 ± 0.03	0.65 ± 0.13	0.63 ± 0.15

Data of CK-MB, total CK, AST and ALT levels, in units per liter (U/L), are presented as means ± SD and were obtained from six (infant) and 17 (adult) animals from each group. Data of heart weight/body weight ratio, in percentage, are presented as means ± SD, and were obtained from six (infant) and 17 (adult) animals from each treatment group. Statistical comparisons were

made using the t-test for CK-MB, ALT and AST/ALT ratio of adult group and the Mann-Whitney test for all other experiments $**p < 0.01$, MTX 6.0 mg/kg vs. control.

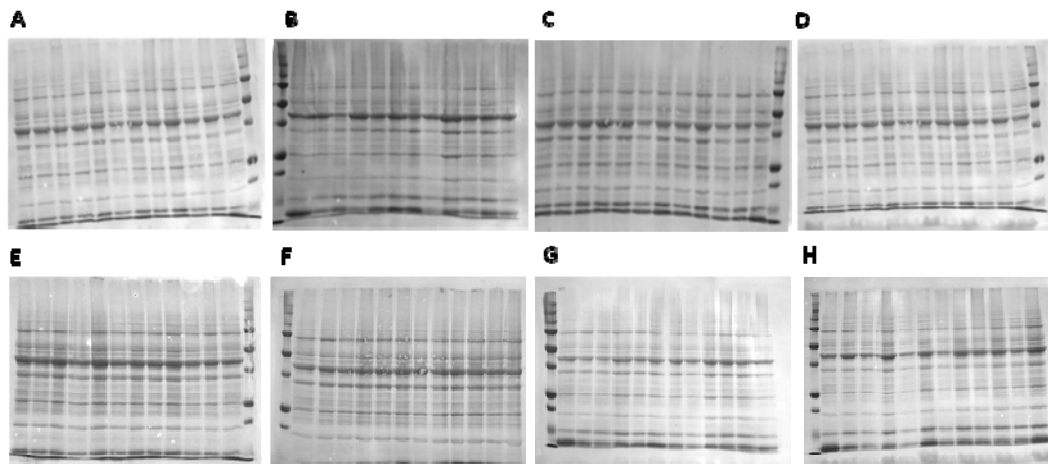


Figure S3. Loading control of Ponceau S staining of [A] ATP synthase subunit beta (52 kDa) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (37 kDa), [B] Superoxide dismutase 2/ manganese-dependent superoxide dismutase (SOD2/MnSOD) (26.6 kDa) and Nuclear kappa B nuclear transcription factor (NF-κB) p65 (60 kDa), [C] catalase (60 kDa) and [D] glutathione peroxidase (22 kDa), [E] Tumour necrosis factor-α (TNF-α) (25 kDa), [F] interleukin-6 (IL-6) (24 kDa), [G] myeloperoxidase (48 kDa), [H] NF-κB p52 (50 kDa) from infant mice exposed to a cumulative dose of 6.0 mg/kg MTX or control animals.

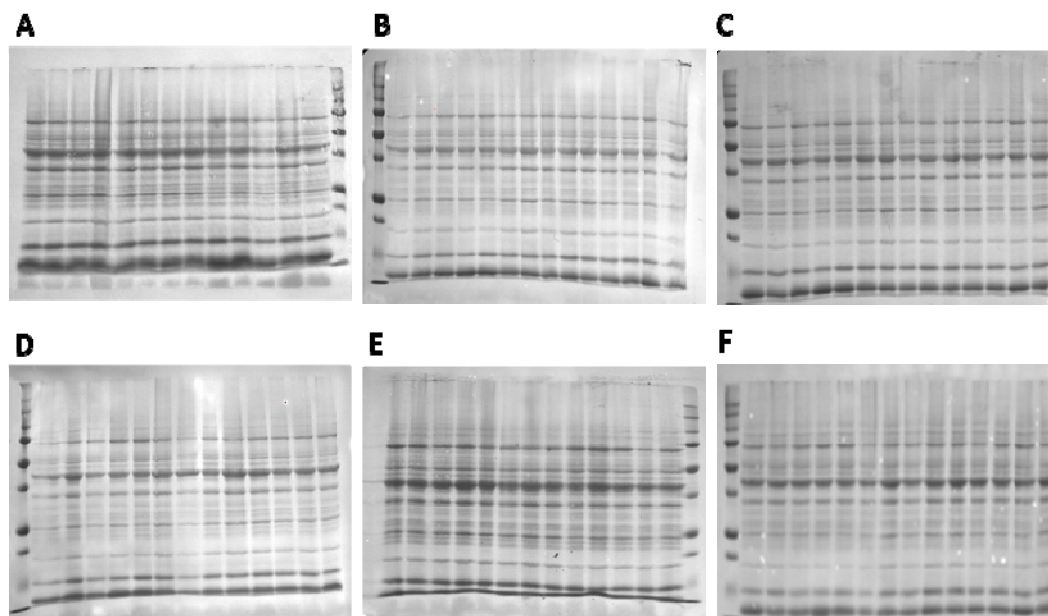


Figure S4. Loading control of Ponceau S staining of [A] ATP synthase subunit beta (52 kDa), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (37 kDa) and interleukin-6 (IL-6) (24 kDa), [B] catalase (60 kDa) and glutathione peroxidase (22 kDa), [C] Tumour necrosis factor-α (TNF-α) (25 kDa), [D] myeloperoxidase (48 kDa), [E] Superoxide dismutase 2/ manganese-dependent superoxide dismutase (SOD2/MnSOD) (26.6 kDa) and Nuclear kappa B nuclear transcription factor (NF-κB) p52 (50 kDa) and [F] NF-κB p65 (60 kDa) from adult mice exposed to a cumulative dose of 6.0 mg/kg MTX or control animals.