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A cautionary note for researchers treating mice with the neurotransmitter norepinephrine

Vance B. Matthews[a,*], Caroline Rudnicka[b], Markus P. Schlaich[a,c]

[a] Dobney Hypertension Centre, School of Medicine and Pharmacology - Royal Perth Hospital Unit, University of Western Australia, Crawley, Western Australia, Australia
[b] Research Centre, Royal Perth Hospital, Perth, Western Australia, Australia
[c] Department of Cardiology and Department of Nephrology, Royal Perth Hospital, Perth, Western Australia, Australia

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ABSTRACT

The sympathetic nervous system plays a crucial role in metabolic function and glucose homeostasis. Norepinephrine is the main neurotransmitter released from sympathetic neurons. The major goal of our studies was to examine the impact of norepinephrine on metabolism related gene expression in obesity *in vivo*. Interestingly, we discovered that norepinephrine had a detrimental effect in our studies.

C57BL6/J mice fed a high fat diet were intraperitoneally injected with 0.2 or 2 mg/kg/day norepinephrine. These doses of norepinephrine have been used previously by other researchers. Survival of the mice was documented. Kidney and bladder tissues were excised and fixed for histological studies.

A subset of norepinephrine treated mice experienced unexpected adverse events which included bladder distension and reduced kidney perfusion as suggested by kidney discolouration. This eventuated in the mice having to be sacrificed or the mice succumbed to the pathological condition. To our knowledge, such an effect of norepinephrine has not been previously reported in mice. Morphological examination of kidney and bladder indicated marked detrimental architectural changes, which we postulate is associated with norepinephrine induced vasoconstriction, urinary retention and renal impairment.

Our studies highlight that administration of norepinephrine to mice may trigger adverse effects relating predominantly to the urogenital tract which can result in decline in a subpopulation of these mice. Researchers administering norepinephrine in mouse models should be aware and look out for these unexpected adverse events associated with the use of norepinephrine.

1. Introduction

Sympathetic hyperactivity is prevalent in obese and type 2 diabetic subjects [1,2], which suggests a critical role for the sympathetic nervous system (SNS) in metabolic function and glucose homeostasis. Our team has illustrated that the SNS may influence glucose control *via* the regulation of glucose reabsorption by sodium glucose co-transporter 2 (SGLT2) expression [3]. We showed that norepinephrine (NE), the main neurotransmitter released from sympathetic neurons which binds to and activates several α- and β-adrenoceptors, increases SGLT2 expression in human proximal tubule cells *in vitro* [3]. Our next aim was to determine whether NE may have effects on metabolism related gene expression *in vivo*. We administered mice with intraperitoneal injections of NE at doses reported by other researchers [4]. We discovered that a subpopulation of NE treated mice experienced a distended bladder phenotype which has not been previously documented. Hence, we caution that animal mortality may be experienced with exogenous administration of NE. Therefore, NE dose and administration timing require careful consideration.

2. Materials and methods

2.1. Animals

Seven week old male C57BL6/J mice were obtained from the Animal Resource Centre (ARC, Perth, Western Australia, Australia). Mice were fed high fat diet (HFD; Specialty Feeds, Glen Forrest, Western Australia, Australia) for 10 weeks to induce obesity as previously conducted by our team [3]. A subset of HFD fed mice were administered NE (Sigma-Aldrich, Sydney, New South Wales, Australia)

Abbreviations: HFD, high fat diet; NE, norepinephrine; SGLT2, sodium glucose co-transporter 2; SNS, sympathetic nervous system

* Correspondence to: Dobney Hypertension Centre, School of Medicine and Pharmacology, Royal Perth Hospital Unit, Level 3, Medical Research Foundation Building, Rear 50 Murray Street, Perth, WA 6000, Australia.

E-mail addresses: Vance.Matthews@uwa.edu.au (V.B. Matthews), Caroline.Rudnicka@uwa.edu.au (C. Rudnicka), Markus.Schlaich@uwa.edu.au (M.P. Schlaich).

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intraperitoneally at a concentration of 2 mg/kg/day (35 mice) or 0.2 mg/kg/day (20 mice) [4]. Norepinephrine was dissolved in 0.9% NaCl (pH = 5.5). Control mice were fed a HFD and administered equal volumes of 0.9% NaCl intraperitoneally. We anticipated to NE treat mice over a 2 week period. However, as soon as the NE administration was observed to be having detrimental health implications such as abdominal swelling, irregular activity, weight loss or postural changes, no further doses were administered and these NE affected mice were prematurely euthanized. Consequently, mice were administered either a single dose of NE at 2 mg/kg/day or 4 doses of 0.2 mg/kg/day to prevent further mortality. All mice had unrestricted access to water. Mice were anaesthetized with methoxyflurane and were euthanized by cervical dislocation. Kidney and bladder tissues were collected and fixed in paraformaldehyde for paraffin wax embedding. Animal ethics approval (R531/15-18) was obtained from the Royal Perth Hospital Animal Ethics Committee.

2.2. Survival analysis

Survival curves were generated using GraphPad Prism 7 software. Mice were classified as a death when they either succumbed to the pathology or had to be euthanised as it was obvious that their health was declining rapidly.

2.3. Histology

Bladder and kidney tissues were sectioned at 5 μm and stained with haematoxylin-eosin (Sigma-Aldrich, Sydney, New South Wales, Australia) to assess NE induced histological changes. Masson's trichrome stain was conducted on kidney tissue sections using standard procedures to evaluate renal fibrosis. Photomicrographs were taken using a Nikon Eclipse Ti Microscope (Nikon Instruments Inc).

3. Results

3.1. Mortality

After treatment of mice with NE, there was 80% survival in mice which were monitored for 2 weeks (Fig. 1). Out of 55 mice, 2 died following one injection of NE and 9 mice had to be euthanised prior to the end of the experiment for ethical reasons.

3.2. Phenotype evaluation

All mice which were detrimentally affected by NE treatment appeared physically unwell, displaying a hunched posture, ruffled coat, slow response to touch and abdominal swelling. Furthermore, they had dark coloured stools. Post-mortem examination of the affected mice indicated no obvious signs of trauma from the intraperitoneal injection. Interestingly, NE affected mice had pronounced bladder distension (Fig. 2A). The urinary bladder was full with discoloured urine, and red blotches appeared on the surface of the bladder which may be symptomatic of glomerulation (Fig. 2A). In comparison, we examined the bladder of all mice that tolerated the limited norepinephrine dosage regime and these bladders appeared normal. We hypothesise that had we treated the mice continuously with norepinephrine, as was originally planned, a much greater proportion of mice, would have developed the distended bladder phenotype.

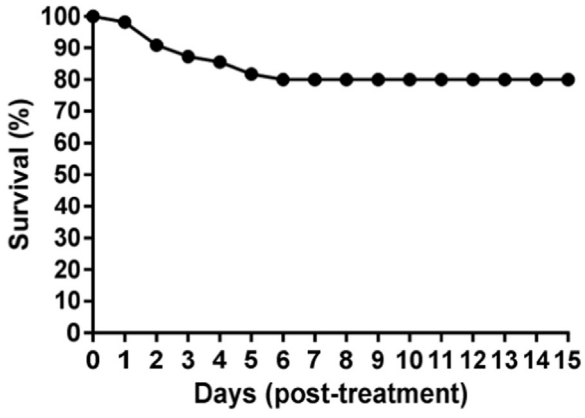

Fig. 1. Effect of norepinephrine on survival in mice. Survival in mice following administration of 2 mg/kg/day or 0.2 mg/kg/day of norepinephrine (n = 55 mice). Results are combined as there were no major differences between 2 mg/kg/day and 0.2 mg/kg/day.

One mouse died several hours after the first NE dose of 2 mg/kg/day. We could expel urine from this mouse with careful pressure, although this was not the case in other mice which were culled or died naturally following NE administration. In addition, NE affected mice often displayed pale kidneys (Fig. 2B). Due to the NE affected mice being so severely effected, we chose not to continue with the gene expression study as mRNA expression may be compromised. We then focussed on collecting mortality and phenotype data.

3.3. Bladder morphology

Bladders from NE affected mice showed folded transitional epithelium, deteriorated lamina propria architecture and a degraded smooth muscle layer (Fig. 2C). These characteristics may reflect bladder dysfunction.

3.4. Kidney morphology

Kidneys from NE affected mice exhibited tubular necrosis, glomeruli destruction and evidence of increased inflammatory cell infiltration (Fig. 3B) compared with kidneys from control mice (Fig. 3A).

Masson's trichrome staining in the kidneys of NE affected mice also demonstrated intense tissue degradation (Fig. 3D). However, we did not observe significant differences in collagen deposition in kidneys from NE affected and control mice (Fig. 3D, C).

4. Discussion

In our study, we administered intraperitoneal injections of NE into HFD fed mice which triggered a decline in a subpopulation of these mice (Fig. 1). The unexpected adverse events with the use of norepinephrine in our current study encouraged us to publish these findings in order to inform other researchers about the potential toxicity of norepinephrine administration in mice and to take appropriate precaution when designing related experiments.

We used previously published concentrations of norepinephrine in our *in vivo* study [4]. However, the previous study was involving acute administration of NE (maximum 4hr treatment time) whereas our study was more of a chronic NE treatment study.

All NE affected mice had a pronounced distended bladder with glomerulation (Fig. 2A). Interestingly, a recent study suggested that norepinephrine induces increased urethral contractility and this may lead to voiding dysfunction in male mice [5]. Studies have reported that athletes may intentionally over distend their bladder prior to competing in a sporting event [6]. This triggers an elevation of the neurotransmitter NE in the blood [7]. Taking our results into consideration, we hypothesise that the elevated NE levels in humans possessing a distended bladder triggers further maintenance of the distended bladder phenotype.

The most common cause of bladder distension is urethral obstruction [8]. In one of our affected mice, we could expel urine forcefully from the distended bladder. This suggests that non-obstructive or partial urinary obstruction may account for the distended bladder

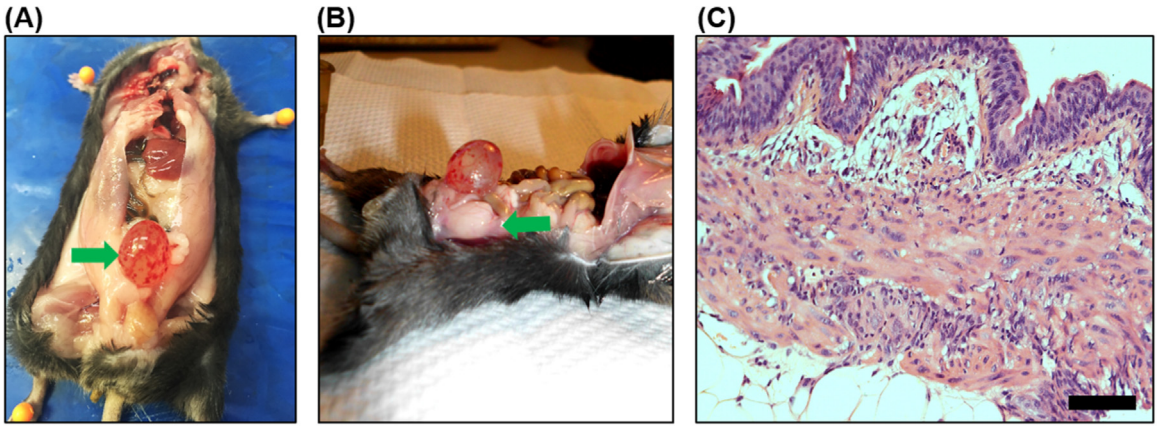

Fig. 2. Effect of norepinephrine on tissue morphology in mice. All norepinephrine treated mice that were detrimentally affected exhibited bladder distension and glomerulations as indicated by the arrow (A), and pale kidneys as shown by the arrow (B). Representative image of hematoxylin-eosin stained distended bladder (C) from mice detrimentally affected by norepinephrine; bar = 100 μm.

phenotype. In comparison, urine could not be forcefully expelled with pressure from the distended bladders of other NE affected mice. It is plausible that obstructive urinary retention may have developed within these mice although we did not formally explore this in our study.

Intriguingly, renal dysfunction is often associated with ureteric obstruction. Acute urinary retention can heighten renal sympathetic activity and promote renal vasoconstriction and impairment [9]. The renal damage we observed in our NE affected mice could be a consequence of NE induced impaired urethral contractions and voiding dysfunction. Urine not being able to pass the urethra may result in compensatory processes in the bladder including enlargement and increased bladder wall thickness due to detrusor smooth muscle hyperplasia and/or hypertrophy [10]. Excessive distension, which could have developed throughout the time course of our experiment, would strain the bladder wall [10]. This may account for our observation that the bladder from NE affected mice had folded transitional epithelium and degraded smooth muscle layers (Fig. 2C). The sustained mechanical pressure that results from bladder distention is likely to have upstream consequences on the kidney, resulting in morphological changes described by us and others [8]. This alters renal hemodynamics, consistent with our evidence that kidneys from NE affected mice being highly vacuolated with inflammatory cell infiltration (Fig. 3). The urethral occlusion and vasoconstrictive effects associated with elevated NE levels may also reduce renal blood flow [11]. In

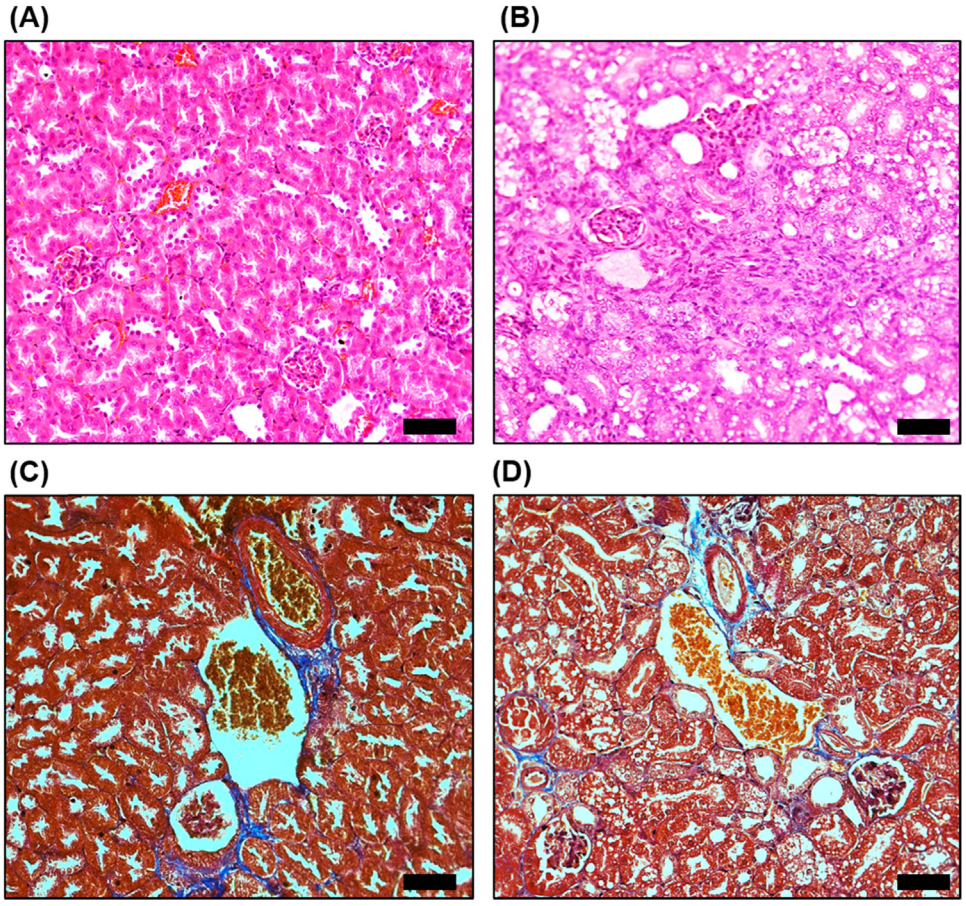

Fig. 3. Norepinephrine affected mice displayed pathology in the kidney. Representative images of hematoxylin-eosin stained kidney from high fat diet fed control mice (A) and NE treated mice that were detrimentally affected (B). Masson's trichrome stained kidney (blue indicates collagen deposition) from high fat diet fed control mice (C) and mice detrimentally affected by NE (D); bar = 75 μm.

concert, these compensatory mechanisms are likely to account for the pale kidney phenotype noticed in our NE affected mice (Fig. 2B).

Many end stage chronic diseases are characterised by organ fibrosis, which can stimulate disordered tissue architecture and organ failure [12]. Fibroblasts are targets of NE and elevated NE is known to contribute to renal fibrosis [13]. We postulated that NE affected mice developed kidney fibrosis. However, we did not observe significant differences in the degree of blue collagen staining in kidneys from NE affected mice compared with controls (Fig. 3D, C). The time course of our experiments may well have been too short for relevant fibrosis to occur.

Future studies should include the use of the specific adrenoceptor blockers prazosin (for α1 receptor), dazoxan (for α2 receptor), metoprolol (for β1 receptor), butoxamine (for β2 receptor) or L-748,337 (for β3 receptor) in concert with norepinephrine treatment. It is anticipated that the adrenoceptor blockers will attenuate or completely prevent the observed NE related adverse events as seen in our study. Secondly, NE and its metabolites could be measured in the treated mice in the future. We believe that the 0.9% NaCl (pH = 5.5) which was used to dissolve NE in our study, should not have resulted in marked NE degradation as supported by other studies [14].

In conclusion, our report provides compelling evidence that norepinephrine administered intraperitoneally at a concentration of 0.2 or 2 mg/kg/day has the potential to cause detrimental effects in mice. The subset of mice which were negatively affected by NE treatment had reduced survival rates, exhibited pale kidneys and a distended bladder phenotype. It is without a doubt that if we had treated the mice continuously with norepinephrine as we had originally planned to do, then a much greater proportion of mice would have developed the distended bladder phenotype. Hence we strongly urge other researchers to carefully consider norepinephrine dosage and administrative timing in their own studies.

Acknowledgements

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Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbrep.2018.08.003.

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