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Oxidative Costs of Reproduction: Oxidative Stress in Mice Fed Standard and Low Antioxidant Diets

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Abstract

Lactation is one of the most energetically expensive behaviours and trade-offs may exist between the energy devoted to it and somatic maintenance, including protection against oxidative damage. However, conflicting data exist for the effects of reproduction on oxidative stress. In the wild, a positive relationship is often observed, but in laboratory studies oxidative damage is often lower in lactating than in non-breeding animals. We hypothesised that this discrepancy may exist because during lactation food intake increases many-fold resulting in a large increase in the intake of dietary antioxidants which are typically high in laboratory rodent chow where they are added as a preservative.

We supplied lactating and non-breeding control mice with either a standard or low antioxidant diet and studied how this affected the activity of endogenous antioxidants (catalase, superoxide dismutase; SOD, and glutathione peroxidase; GPx) and oxidative damage to proteins (protein carbonyls, PC) in liver and brain tissue. The low antioxidant diet did not significantly affect activities of antioxidant enzymes in brain or liver, and generally did not result in increased protein damage, except in livers of control mice on low antioxidant diet. Catalase activity, but not GPx or SOD, was decreased in both control and lactating mice on the low antioxidant diet. Lactating mice had significantly reduced oxidative damage to both liver and brain compared to control mice, independent of the diet they were given.

In conclusion, antioxidant content of the diet did not affect oxidative stress in control or reproductive mice, and cannot explain the previously observed reduction in oxidative stress in lactating mammals studied in the laboratory. The reduced oxidative stress in the livers of lactating mice even under low antioxidant diet treatment was consistent with the ‘shielding’ hypothesis.

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Introduction

The evolution of life histories has been explained by the presence of limited resources, which result in a trade-off between survival (somatic maintenance) and reproduction [1, 2]. The existence of a trade-off implies that resources are limited, or the capacity to utilise resources is physiologically constrained (e.g. [3-6]). This may be particularly important during periods when energy demands are high, such as during lactation, during which mammals appear constrained by their capacity to dissipate heat [7-10]. It has been suggested that reproduction may lead to an increase in oxidative stress thus affecting ageing (Oxidative stress theory of ageing, [11], but see [12, 13]). Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the capacity of the antioxidant protection and repair systems. ROS are primarily produced in mitochondria during the process of oxidative phosphorylation when oxygen can react prematurely with an electron to form the superoxide anion. ROS is deleterious to an organism as it can cause damage to macromolecules, i.e., DNA, proteins and lipids. ROS can be removed by exogenous antioxidants obtained via the diet (e.g. carotenoids, Vitamin C and E), and by endogenously produced antioxidants, i.e., superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx). Some ROS escape detoxification and cause damage, which may be repaired (DNA) or removed by turnover (proteins), but some damage accumulates leading to an increasing risk of malfunction.

It has been hypothesised that during reproduction selective resource allocation reduces investment in protection and repair, resulting in an increase in oxidative damage, reducing survival and fecundity. Many studies attempting to determine if oxidative stress is the proximal cost of reproduction produced ambiguous results [14-25]. Supportive evidence has been found in birds and mammals, e.g., a study investigating changes in antioxidant activity in response to brood size manipulations in zebra finches showed reduced antioxidant protection in serum with increased reproductive effort [14]. Recently, a study in the North American red squirrel found increased oxidative damage to serum proteins during lactation [21], which was reduced when populations were given supplementary feeding. Contradictory results have also been found, showing no or negative relationships between oxidative stress and reproductive effort, e.g. oxidative damage was found to be reduced in the livers of reproductive compared to non-reproductive female house mice [22, 26] and bank voles [24] and breeding and non-breeding zebra finches did not differ in their resistance to oxidative stress (include tissue) [17], although a negative relationship between the number of eggs laid and oxidative resistance was found in that same study.

The cause of the variation in responses has been debated [12, 13, 27] and various suggestions explaining the discrepancies have been offered, including differences in experimental design, biological markers, tissues, species, laboratory vs. field conditions. Indeed, depending on the marker used or the tissue measured contradicting results can be found within the same individuals [28, 29]. One possibility for the lowered damage during reproduction observed noticeably in lactating rodents' livers in captivity ([22, 24]) is that this does not reflect a change in protection strategy but may rather be an artefact of elevated food intake in lactation [30] combined with the very high levels of antioxidants that are added to rodent chow as a preservative (Speakman et al. submitted). During lactation in

mice at room temperature, food intake increases by about a factor of 4 to 5 reaching a plateau around day 10-18 of lactation (Johnston et al., 2001). In laboratory conditions, where animals are fed on standardised diets, the intake of exogenous antioxidants thus increases proportionately to food intake. This increased intake of exogenous antioxidants may mitigate detrimental effects of increased ROS production during peak lactation, and may explain why negative effects of lactation on oxidative stress in the liver have been observed [22, 24]. Since the liver is the first organ to experience such absorbed antioxidants it would be particularly prone to such impacts, possibly explaining why damage to the liver often goes down in such studies [22, 24, 28, 29] when damage to other tissues goes up [24, 28, 29]. To test this hypothesis we formulated a low antioxidant diet that had 5-times less antioxidants compared to the standard diet. At peak lactation therefore intake of antioxidants of lactating mice on the low antioxidant diet would thus be similar to the intake of antioxidants in non-reproductive control animals on the standard diet. Hence, if endogenous antioxidant overload was an issue, these animals would be expected to have increased oxidative stress noticeably in their livers, compared to both lactating and control mice fed the standard diet.

Materials and Methods

Animals & Housing

Sixty-five MF1 mice, 15 male and 50 female, were obtained from Harlan laboratories (Bicester, UK) at 10 weeks of age, and housed in shoebox cages (NKP Cages, M3 Mouse Cage, 48"×15"×13") with sawdust shavings and paper wool for bedding. After 2 weeks of acclimation, mice had *ad libitum* access to food (CRM(P) standard laboratory chow) and water and were housed under a 12:12 LD cycle and 21°C (±1°C). We used MF1 mice because of our extensive prior knowledge of its reproductive energetics [31].

Experimental protocol

Female mice were randomly assigned to one of 4 groups ensuring an even body weight distribution: 1. Control on Standard diet (n=11), 2. Control on Low antioxidant diet (n=10), 3. Reproductive mice on Standard diet (n=15) or 4. Reproductive mice on Low antioxidant diet (n=15). The low antioxidant diet (D12072502) used was specifically formulated by Research Diets (New Brunswick, USA) for this study based on the D12450B diet (10%, 70% and 20% of kJ from fat, carbohydrates and protein respectively) with a reduced antioxidant content by removing Vitamin E from the vitamin mix and using a different type of fat source that did not contain antioxidants, i.e., tocopherol-stripped corn oil was used in the low antioxidant diet as the main fat source instead of soybean oil and lard that may contain some antioxidants. See *Table 1* for detailed dietary content and Supplementary Table 1 for a detailed description of the vitamin mixes used.

Reproductive mice were housed in pairs (one each from the standard and low antioxidant diet groups) with a single male for 7 days to induce pregnancy. When the male was removed both females were housed individually for the remainder of the study. Control mice were housed individually from the start of the experiment and not exposed to a male mouse. Reproductive mice were fed standard lab chow (CRM(P)) until day 1 of lactation (day 0 =

date of birth), when the new diet, with Standard or Low antioxidants was introduced. The new diet was introduced initially as a 50-50% mix of CRM(P) and the assigned diet, and from day 2 till the end of lactation only the assigned diet was provided. Control mice were introduced to the new diets in the same way on the first day of the first reported lactation in reproductive mice. Food intake and body mass were measured daily throughout the experimental period, except on the day of parturition (day 0: [8]). In addition, litter mass and litter size was recorded from day 1 of lactation onwards in reproductive mice. On day 18 of lactation all mice were culled by cervical dislocation and tissue samples (brain, liver, and brown adipose tissue(BAT)) were dissected out and stored in a -80°C freezer until analysis. Three animals that did not give birth successfully or lost their litter before day 18 of lactation were removed from the study, reducing the sample size in the breeding standard and low antioxidant diet groups to 14 and 13 respectively.

Antioxidant Enzyme Activity Measurements

Frozen samples were homogenised in 50mM phosphate buffer and centrifuged at 4000g for 20 min at 4°C . The supernatant fraction was transferred to 1.5ml Eppendorf tubes and stored on ice or at -80°C until analysis of antioxidant activity. Catalase activity was always assessed in freshly homogenised supernatants. Supernatant was treated with 1% Triton X-100 and activity of catalase was assessed by detecting the amount of H_2O_2 which was decomposed by catalase activity at 25°C using KMnO_4 at 240 nm, based on the method detailed by Cohen et al., 1970. One unit of catalase was defined by the decrease of $1\mu\text{mol}$ of H_2O_2 per minute. Superoxide dismutase activity (SOD) was followed spectrophotometrically at 340nm by the inhibition of the auto-oxidation of pyrogallol by SOD at 25°C using the method described by Marklund and Marklund (1974). One unit of SOD activity was defined as the amount of enzyme that causes a 50% inhibition of pyrogallol auto-oxidation. Glutathione peroxidase catalyses the conversion of glutathione (GSH) to oxidised glutathione (GSSG). GSSG is then converted back to its reduced form in a reaction catalysed by glutathione reductase (GR). This reaction oxidises NADPH to NADP^+ which can be monitored spectrophotometrically at 340 nm giving a measure of the activity of GPx, as described by Paglia and Valentine (1967) and Lawrence and Burk (1976). To correct for spontaneous reactions that occur in the absence of the enzyme, background reactions were run without the presence of the sample and subtracted from the assay values. One unit of GPx is defined as the amount of enzyme that oxidises $1\mu\text{mol}$ of NADPH per min. All enzyme activities were corrected for the protein concentration of the individual tissues as determined by the Bradford assay (Bio-Rad).

Oxidative damage (protein carbonyls)

Oxidative damage to proteins was assessed in liver and brain homogenates by measuring the quantity of protein carbonyls in a protein sample following derivatisation of proteins with dinitrophenylhydrazine (DNP, Protein carbonyl enzyme immune-assay kit, BIOCELL Corporation Ltd., New Zealand). Before the analysis protein concentration for each sample was determined using the Bradford assay to calculate the amount of sample required in the test (200-300 μg of protein).

Statistical Analysis

All data were normally distributed (Kolmogorow-Smirnov test in SPSS v17.0) and parametric tests were used throughout. Repeated measures GLMs were used to test for differences between the group/diets in the development of body mass, food intake, litter mass and litter size (RM ANOVA). MIXED models with body mass or litter size as time-variant covariates were used where appropriate as described in the text. Two-way ANOVA (Univariate GLM in SPSS with group (control vs. lactating) mice, and diet – (standard vs. low antioxidants) as fixed factors) were performed to test for differences between reproductive status and diet groups in the level of antioxidant protection and oxidative damage. Post-hoc t-tests were performed when a significant effect was found to pinpoint which groups and at which time points significant differences were present. All tests were two-tailed and statistical significance was set at $p = 0.05$.

Results

Reproductive performance

As expected, body mass of reproductive mice increased steeply during pregnancy (*Figure 1A*). During lactation, reproductive mice maintained a slightly increased body mass compared to control mice, but no significant effect of antioxidant diet was observed (*Figure 1A*, RM ANOVA day 2-18, Time: $F_{15,675}=4.7$, $p<0.001$, Diet: $F_{1,45}=0.1$, $p=0.88$, Group: $F_{1,45}=50.0$, $p<0.001$ and Group x Diet: $F_{1,45}=0.4$, $p=0.61$). A significant interaction between diet and time was also observed (Diet x Time: $F_{15,675}=10.8$, $p<0.001$) and closer inspection indicated that reproductive mice on the low antioxidant diet had slightly reduced body mass compared to those on the standard diet on day 2 and increased body mass on days 15-18 of lactation (post-hoc t-tests, $p<0.05$). Body mass of the control mice on different diets did not differ significantly at any point.

Food intake was significantly increased throughout the period of lactation, but did not differ significantly between mice on the standard or low antioxidant diets (*Figure 1B*, RM ANOVA day 2-18, Time: $F_{15,675}=17.5$, $p<0.001$, Diet: $F_{1,45}=0.1$, $p=0.76$, Group: $F_{1,45}=1406.0$, $p<0.001$ and Group x Diet: $F_{1,45}=0.3$, $p=0.59$). A significant interaction between diet and time was again observed (Diet x Time: $F_{15,675}=5.1$, $p<0.001$) and post-hoc t-tests revealed significant differences in food intake between lactating mice on the different diets on days 2 when mice on low antioxidant had reduced intake and on day 13 and 17 when their food intake was significantly increased (post-hoc t-tests, $p<0.05$). No significant differences in food intake were found between control mice on the two diets.

Litter mass was significantly higher in mice on the low antioxidant diet compared to mice on standard diet up until day 12 of lactation (*Figure 2A*, independent t-tests, $p<0.05$). At birth, litter mass was 25.2 ± 3.9 g and 19.6 ± 4.4 g in reproductive mice on the low and standard diets respectively (t-test, $p=0.002$), increasing to 110.1 ± 12.2 g and 107.5 ± 13.9 g at time of weaning (day 18, $p=0.61$). Litter size also differed significantly between groups and was 13.1 ± 2.7 and 9.8 ± 3.0 at birth and 12.7 ± 2.1 vs. 9.8 ± 3.0 at day 18 in mice on low and standard antioxidant diets respectively (t-test, day 0, $p=0.006$, day 18, $p=0.008$). Note though that animals only received their assigned diets from day 2 of lactation onwards, so

any differences in litter mass or size at birth was not due to a diet effect. Litter size was significantly correlated with litter mass (*Figure 2B*, $r=0.24$, $p<0.001$), and when litter mass data were corrected for litter size no significant difference in litter mass between mice on the low and standard antioxidant diets remained (MIXED model with litter size as time-variant covariate, Diet group: $F_{1,27}=0.61$, $p=0.44$, Day: $F_{17,423}=1235.2$, $p<0.001$, Diet x Time: $F_{17,423}=1.6$, $p=0.055$).

Liver antioxidants

Catalase activity in the liver was not significantly different between lactating and control mice, or between mice fed standard or low antioxidant diets (*Figure 3A*, GLM, Group: $F_{1,47}=2.6$, $p=0.11$, Diet: $F_{1,47}=1.2$, $p=0.28$). A significant interaction between group and diet was found (*Figure 3A*, $F_{1,47}=4.1$, $p=0.05$) indicating that reproductive mice responded differently to the diet. However, post-hoc tests showed no significant differences between any of the groups. SOD activity was not affected by reproductive status or diet (*Figure 3B*, GLM, Group: $F_{1,47}=0.9$, $p=0.36$, Diet: $F_{1,47}=0.5$, $p=0.51$, Group x Diet: $F_{1,47}=0.4$, $p=0.51$). Overall, GPx activity was significantly lower in lactating mice compared to control mice, but diet did not affect GPx activity (*Figure 3C*, Group: $F_{1,47}=5.8$, $p=0.02$, Diet: $F_{1,47}=0.8$, $p=0.38$, Group x Diet: $F_{1,47}=0.6$, $p=0.45$). Post-hoc t-test within each diet did not indicate a significant effect of group though. Oxidative damage measured by the presence of protein carbonyls was significantly increased in control mice compared to reproductive mice and was significantly increased on the low antioxidant diet (*Figure 3D*, Group: $F_{1,47}=16.7$, $p<0.001$, Diet: $F_{1,47}=40.3$, $p<0.001$, Group x Diet: $F_{1,47}=10.2$, $p=0.003$). The significant interaction further shows that control and reproductive mice differed in their responses to the diets. As is clear from *Figure 3D* control mice had increased levels of protein carbonyls on the low antioxidant diet compared to the standard diet, whereas protein carbonyls did not differ significantly between reproductive mice on the low antioxidant diet vs. the standard diet.

Brain antioxidants

Catalase activity was not significantly affected by reproduction or diet (*Figure 4A*, GLM, Group: $F_{1,47}=2.3$, $p=0.13$, Diet: $F_{1,47}=0.01$, $p=0.93$, Group x Diet: $F_{1,47}=0.4$, $p=0.51$). SOD activity was significantly increased in reproductive mice compared to control mice, but was not affected by diet (*Figure 4B*, GLM, Group: $F_{1,47}=7.4$, $p=0.01$, Diet: $F_{1,47}=2.8$, $p=0.10$, GroupxDiet: $F_{1,47}=0.3$, $p=0.58$). GPx activity was not significantly affected by group or diet (*Figure 4C*, Group: $F_{1,47}=1.8$, $p=0.18$, Diet: $F_{1,47}=3.5$, $p=0.066$, GroupxDiet: $F_{1,47}=0.6$, $p=0.44$). Oxidative damage in the brain was significantly decreased in reproductive mice compared to control mice, but was not affected by diet (*Figure 4D*, Group: $F_{1,47}=8.5$, $p=0.006$, Diet: $F_{1,47}=2.6$, $p=0.11$, Group x Diet: $F_{1,47}=0.1$, $p=0.78$).

Discussion

Oxidative stress has been hypothesised to be the proximal factor explaining the costs of reproduction within the resource allocation frame-work [14-25]. Within this framework it is expected that reproduction leads to increased levels of oxidative stress, which may have detrimental effects on future reproductive success and survival. Studies investigating these

interactions have shown contradicting results (see [32] for a recent review). Several studies in birds have confirmed a negative role of reproduction on oxidative stress (e.g., reduced antioxidant capacity or increased oxidative damage). For instance, zebra finches show decreased resistance to oxidative stress (i.e., reduced antioxidant activities) with increased clutch size [14, 17]) and birds that engaged in a higher number of breeding events had a weaker red blood cell resistance to oxidative stress which was correlated to an increased risk of mortality [15]. In mammals, increased oxidative damage has been reported in field studies on lactating female eastern chipmunks [33], and female American red squirrels [21], but in wild Soay sheep no relationship was found between reproduction and oxidative damage [25]. Several lab-based studies in mice and voles have shown reduced levels or no changes to oxidative damage in lactating animals compared to non-reproductive animals [22, 24]. We hypothesised that captive small rodents may not experience the anticipated increased levels of oxidative stress, particularly in their livers, because the intake of antioxidants in their food (which is abnormally high when compared with free-living animals) is greatly increased during lactation, neutralising any increase in ROS production or decrease in investment in enzymatic protection. The fact that damage is most often reported to be reduced in the liver [22-24, 26], compared with changes in other tissues, is consistent with this hypothesis, as the liver would be the first organ where such absorbed antioxidants would be received after transport through the portal vein. Here, we studied the effects of a low antioxidant content in the diet on oxidative stress control and reproductive mice. We predicted that if dietary antioxidants cause the reduced damage observed in lactating mice, then lactating mice on low antioxidant diets would have greater oxidative damage than would lactating mice on the standard diet. Moreover, they would have similar exogenous antioxidant protection to mice on standard diets, but should reduce investment into endogenous enzymatic antioxidants (disposable soma theory: [34]) and hence have increased oxidative damage relative to the control mice.

Changes in body mass and food intake were similar in mice on the low and standard antioxidant diets throughout the course of lactation, although body mass was slightly reduced in reproductive mice on the low antioxidant diet compared to the standard diet towards the end of lactation. The reason for this decrease in body mass was unclear and a similar decrease was not observed between control mice on the two diets. Importantly, no significant differences in litter mass were observed between mice fed low and standard antioxidant diets when corrected for litter size, showing that both groups showed a similar investment in their litters. Litter size was significantly reduced in mice fed the standard diet compared to low antioxidant fed mice, but since litter size is established during pregnancy, when both groups were on the same diet, this was unrelated to an effect of the diet.

In contrast to the prediction, oxidative damage in liver and brain was unchanged in lactating mice on the low antioxidant diet compared to control mice on the standard diet. In fact, the antioxidant diet did not significantly affect any of the variables except oxidative damage to the liver, which was increased in controls on the low antioxidant diet compared to controls on the standard diet. Moreover, low statistical power is not a likely explanation for our results; i.e., we had 80% power to detect a 30% difference in oxidative damage levels, which was approximately the difference found in previous studies in lactation [22].

Reproduction did affect antioxidant enzyme activities and oxidative damage in some tissues. In agreement with previous studies in rodents [22-24, 26], reproductive mice did not have increased oxidative damage compared to control mice indicated by reduced levels of protein carbonyls in the liver and brain. Studies including assays of lipid and DNA damage have found similar results [22-24, 26]. Oxidative stress results from an imbalance between oxidative protection and production of ROS and one may speculate that increased oxidative stress was not observed in reproductive animals, because ROS production was reduced or antioxidant protection was upregulated during reproduction. Previous studies have shown increases in activity of catalase [23], SOD [26], or increased levels of glutathione [22] in livers of reproductive mice. We found an increase in SOD activity in the brain in reproductive compared to control mice, but a reduction in GPx activity in liver. These results once more point out the complexities of the relationship between antioxidant protection and oxidative damage and the variability of these responses between tissues of interest [28, 29] and cannot explain the lack of a negative relationship between reproduction and oxidative stress.

A recent meta-analysis of the literature has shown that increased reproductive effort (i.e., increased clutch, litter or brood size) generally results in increased oxidative damage. However, when comparing reproductive to non-reproductive animals reproduction is associated with a reduction in certain markers of oxidative damage particularly in liver, heart and ovarian follicles [32]. This led to the hypothesis that the transition to the reproductive state triggers a pre-emptive reduction in levels of oxidative damage in order to shield mothers, their gametes and developing offspring from oxidative stress resulting from increased reproductive effort [32]. This idea of oxidative shielding is in agreement with our results showing no change or even a reduction in oxidative damage in lactating compared to non-reproductive mice.

In conclusion, reducing the amount of antioxidants in the diet of control and reproductive mice did not significantly affect oxidative stress experienced, and cannot explain why most laboratory studies have not been able to show an oxidative cost of reproduction. These results agree with the idea of oxidative shielding [32].

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Figure 1.

Body mass and food intake during pregnancy and lactation in reproductive (grey symbols) and control (white symbols) mice on standard (circles) or low (triangles) antioxidant diets. Values are shown as means $\pm$ sem.

Figure 2.

A. Litter mass during lactation in reproductive mice fed standard or low antioxidant diets.

Values are shown as means $\pm$ sem. B. Correlation between litter size and litter mass at weaning (day 18) in reproductive mice fed Standard (solid trend line, $r=0.27$, $p<0.001$) or Low antioxidant (dashed trend line, $r=0.12$, $p=0.065$) diets.

Figure 3.

Antioxidant enzyme activity and oxidative damage in the livers of reproductive and control mice on standard (S) or low (L) antioxidant diets. (A) Catalase activity, (B) Superoxide dismutase activity, and (C) Glutathione peroxidase activity (D) Protein carbonyls. Different letters inside the bars represent significant differences between the groups (post-hoc tests). Values are shown as means $\pm$ sem.

Figure 4.

Antioxidant enzyme activity and oxidative damage in the brains of reproductive and control mice on standard (S) or low (L) antioxidant diets. (A) Catalase activity, (B) Superoxide dismutase activity, and (C) Glutathion peroxidase activity (D) Protein carbonyls. Different letters inside the bars represent significant differences between the groups (post-hoc tests). Values are shown as means $\pm$ sem.

Table 1

Nutritional information for standard and low antioxidant diet

Product #	Standard D12450B		Low antioxidant D12072502	
	g	kJ	g	kJ
Protein	19%	10%	19%	10%
Carbohydrate	67%	70%	67%	70%
Fat	4%	20%	4%	20%
Total (kJ/g)	15.9	100%	15.9	100%
Soybean oil	25	941	0	0
Corn oil, tocopherol stripped	0	0	45	1694
Lard	20	753	0	0
Mineral Mix S10026	10	0	10	0
DiCalcium Phosphate	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0
Potassium Citrate, 1 H ₂ O	16.5	0	16.5	0
Vitamin mix V10001	10	167	0	0
Vitamin mix V13401 (- VitE)	0	0	2	33
Choline Bitartrate	2	0	2	0

Diets were formulated by Research diets, New Brunswick, USA. g = weight in grams and kJ shows energy content in kJ.