Caloric restriction increases free radicals and inducible nitric oxide synthase expression in mice infected with Salmonella Typhimurium

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Synopsis

It is well known that CR (caloric restriction) reduces oxidative damage to proteins, lipids and DNA, although the underlying mechanism is unclear. However, information concerning the effect of CR on the host response to infection is sparse. In this study, 6-month-old mice that were fed AL (ad libitum) or with a CR diet were infected with Salmonella serovar Typhimurium. EPR (electron paramagnetic resonance; also known as ESR (electron spin resonance)) was used to identify FRs (free radicals). These results were subsequently correlated with SOD (superoxide dismutase) catalytic activity, iNOS [inducible NOS (nitric oxide synthase) or NOSII] expression and NO (nitric oxide) content. EPR analysis of liver samples demonstrated that there was a higher quantity of FRs and iron–nitrosyl complex in infected mice provided with a CR diet as compared with those on an AL diet, indicating that CR was beneficial by increasing the host response to Salmonella Typhimurium. Furthermore, in infected mice on the CR diet, NOSII expression was higher, NO content was greater and spleen colonization was lower, compared with mice on the AL diet. No changes in SOD activity were detected, indicating that the NO produced participated more in the formation of iron–nitrosyl complexes than peroxynitrite. These results suggest that CR exerts a protective effect against Salmonella Typhimurium infection by increasing NO production.

Key words: electron paramagnetic resonance (EPR), iron–nitrosyl complex, nitric oxide, superoxide dismutase (SOD)

INTRODUCTION

It has been demonstrated that CR (caloric restriction), which involves a reduction in caloric intake while maintaining adequate nutrition and normal functions of the organism, decreases production of ROS (reactive oxygen species) [1,2] by preserving antioxidant enzyme expression and catalytic activity [3–5]. As most studies related to oxidative injury and CR have been performed on isolated tissues from non-stressed animals [4] there is little information regarding the ability of animals experiencing CR to respond to microbial infections, such as with Salmonella serovar Typhimurium.

The reports in the literature regarding the effects of CR on host defenses against infection in animals and humans are contradictory. Notably, the studies in which CR enhanced the host defences and resistance to infection were conducted with long-term CR [6–10], while those in which CR reduced host defences and resistance to intact pathogens were done with short-term CR [11,12].

It is well known that the immune response against infection by a micro-organism includes production of ROS [such as O$_2$•− (superoxide radical)] [13] and RNS (reactive nitrogen species) [14]. In recent years, one form of RNS, NO (nitric oxide), has received great attention because of its important role in the host defence against a wide variety of microbes [15–18]. Because NO is formed in small amounts in vivo and is rapidly destroyed by interaction with oxygen, its measurement is difficult. However, NO can react with iron from proteins to form iron nitrosyl complexes that yield characteristic EPR or ESR signals [19]. For this

Abbreviations used: AL, ad libitum; ALC, ad libitum control; CFU, colony-forming units; CR, caloric restriction; CRC, CR control; DNIC, dinitrosyl–iron complex; FR, free radical; IFN-γ, interferon-γ; NOS, nitric oxide synthase; iNOS, inducible NOS; INT, 2-(4-indophenyl)-3(4-nitrophenyl)-5-phenyl tetrazolium chloride; O$_2$•−, superoxide radical; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF-α, tumour necrosis factor-α.

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reason, EPR is one of the most widely used methods for detecting NO.

NO is produced when L-arginine is transformed by the catalytic action of NOS (nitric oxide synthase). One isoenzyme of NOS is iNOS (inducible NOS or NOSII), which shows increased expression in mice infected with Salmonella Typhimurium. This biological property is considered a defence that an organism requires to avoid Salmonella Typhimurium proliferation in infected organs [20]. There is increasing evidence that O$_2$•¯ interacts with NO [15,21] to yield a highly reactive product, peroxynitrite (ONOO$^-$), which exhibits extremely cytostatic/cytotoxic and, consequently, pathogenic effects [22–24]. Therefore the intracellular killing of Salmonella Typhimurium is dependent on both ROS and RNS [25,26].

The aim of the present study was to determine the effect of CR on FR (free radical) content and NOSII expression in liver and skeletal muscle tissue of mice fed either AL (ad libitum) or with a CR diet and subjected to Salmonella Typhimurium infection.

**MATERIALS AND METHODS**

**Animals**

A total of 150 2-month-old male Balb/c mice were separated into six experimental groups ($n = 25$ per group). Animals were housed individually and maintained in a 12 h:12 h (light/dark) cycle at 18–22°C with free access to water, and were fed an NIH 31 diet (Harlan Laboratories). This diet is used at the National Institutes of Health as the standard reference diet for biological and biomedical research and is widely used for commercial rat and mouse production colonies. Three groups were fed AL, and another three groups were subjected to 40% CR with the NIH 31 diet. The content of l-arginine was 1.28% of the total diet and nitrite and nitrate were not present. After 4 months, when the animals were 6-months old, two AL and two CR groups were infected with Salmonella Typhimurium. The remaining groups of mice remained uninfected as controls [ALC (ad libitum control) and CRC (CR control)]. One of each of the infected AL and CR groups was killed on day 7 after Salmonella Typhimurium was inoculated (ALS-7 and CRS-7) and the other two groups were killed on day 14 (ALS-14 and CRS-14). Animals were treated according to the protocol approved by the Ethics and Institutional Animal Care and Use Committees from the Escuela Superior de Medicina, IPN.

**Salmonella Typhimurium infection**

The bacterial strain A.T.C.C. 14028 was cultivated in brain heart infusion broth (Difco) for 18–24 h at 37°C under agitation. Bacteria were harvested at the end of the exponential growth phase and used for animal inoculation.

Mice were pretreated orally with 1 ml of 0.1 M sodium bicarbonate solution and then orally infected with a sublethal dose of 10$^7$ CFU (colony-forming units) of Salmonella Typhimurium through a plastic tube. Mice were monitored twice daily, and the percentage of survivors was recorded [27]. Freshly voided faecal pellets from each infected mouse were collected and weighed daily during the 7 or 14 days post-infection to estimate bacterial elimination based on the plate count method described below.

**Splenic colonization**

To determine the in vivo persistence of Salmonella Typhimurium, mice were killed under diethyl ether anaesthesia at day 7 or 14 post-inoculation. Spleens were removed aseptically and placed in 1 ml of sterile ice-cold PBS containing 0.1% (v/v) Triton X-100. Spleen homogenates were serially diluted in PBS and plated on to SS agar, and incubated overnight at 37°C. Animals were anaesthetized with diethyl ether and killed by cervical dislocation. Liver and skeletal muscle tissues were quickly removed and cut into pieces. Liver samples (weighing 100 ± 15 mg) and muscle samples (weighing 50 ± 15 mg) were placed in EPR tubes, which were maintained at −80°C until measurements were made.

**EPR spectroscopy**

The EPR measurements were carried out at 77 K using a JEOL JES-RES 3X spectrometer operating at 100 KHz field modula. EPR spectra were recorded at X-band frequency (9.15 GHz), at 323 ± 20 mT field centre and sweep, 1 mW microwave power, 0.03 s time constant, 120 s acquisition time, 0.126 mT modulation amplitude and $2 \times 10^3$ receiver gain. The EPR spectra were recorded in digital form (an average of three scans was used for the working spectrum). The number of paramagnetic species contained in the samples was obtained by double integration of EPR signals and normalized with the weight of each sample ($n = 3$). The g values were calculated from the measurements of the magnetic field with a resolution of ±0.01 mT using microwave frequency parameters and DPPH (2,2-diphenyl-1-picrylhydrazyl) as a marker (g = 2.0037) [28]. DNIC (dinitrosyl-iron complex) with cysteine and Hb–NO complexes were obtained as described elsewhere [19,29].

**Nitrate/nitrite determination**

Liver and skeletal muscle tissues were homogenized (100 and 50 ± 15 mg respectively) in 1 ml of PBS with Milli-Q water. The homogenate was centrifuged at 21 000 g for 20 min at 4°C, and aliquots of the supernatant were either stored at −20°C for total protein determination by the Lowry et al. method [30] or to calculate nitrite levels using a nitrate/nitrite colorimetric assay kit (Cayman Chemical). First, nitrate was converted into nitrite by using nitrate reductase enzyme. Afterwards, nitrite was converted into an azo compound by the addition of Griess reagent [31]. The absorbance of this compound was determined at 540 nm by employing a microplate reader (Benchmark; Bio-Rad Laboratories).
The absorbance was considered the sum of nitrates and nitrites, representing total NO.

**RNA extraction and cDNA synthesis**

To study hepatic NOSII expression, total RNA from liver was extracted using the Perfect RNA™ Eukaryotic Mini kit (Eppendorf). Isolated RNA was quantified using the GENESYS™ 10 Series (ThermoSpectronic) and 5 μg of RNA was separated on a 1.0% agarose gel containing ethidium bromide in Mops buffer. To prevent DNA contamination, RNA samples were treated with amplification grade DNase I (Invitrogen) before reverse transcription. All RNA samples were stored at −70 °C in RNA elution solution until further use. A total of 500 ng of RNA was reverse-transcribed in a 20 μl reaction volume using Transcripter First Strand cDNA Synthesis Kit (Roche), and the reactions were conducted in a Mastercytcle thermocycler (Eppendorf). The protocol was based on the manufacturer’s recommendations.

**Real-time quantitative PCR assay**

The amplified cDNA was quantified on a photometer at 260 nm. Real-time PCR was carried out using the Rat Universal ProbeLibrary (Roche Diagnostics). Specific oligonucleotide primers were originally generated by using the online assay design software (ProbeFinder: http://www.roche-applied-science.com/sis/rtpcr/upl/index) and the primer sequence for NOSII was 5′-GCTACACCTCACAACGCAAC-3′ (sense) and 5′-TTCTTGGCGTGTTGATGCCTC-3′ (anti-sense). The 20 μl reaction mixture contained 1× LightCycler TaqMan Master reaction mixture (Roche Diagnostics), 200 nM of each primer, 100 nM Universal ProbeLibrary probe, 0.5 unit of LightCycler Uracil-DNA Glycosylase and 2 μl of standard DNA in the appropriate dilution. The amplification was performed in borosilicate glass capillaries (Roche Diagnostics). Samples were normalized using the housekeeping gene, 18S.

**SOD (superoxide dismutase) activity**

The SOD level was estimated by using an SOD assay kit (Fluka). Xanthine and xanthine oxidase were used to generate the superoxide anion, which reacted with INT [2-(4-indophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride] to form a red formazan dye [32]. One enzyme unit of SOD is defined as the amount that inhibits the INT reaction by 50%. Specific activities are defined as units/mg of protein.

**Statistical analysis**

Results are presented as means ± S.E.M. The comparison of two groups was analysed using an unpaired two-tailed Student’s t test. For comparisons among multiple groups, one-way ANOVA followed by a Holm–Sidak test was employed. P < 0.05 was considered statistically significant. All analyses were performed using the statistical software program Sigma Stat for Windows Version 2.03 (SPSS). The statistical significance of the difference between Kaplan–Meier survival curves was tested with the log-rank test [33].

**RESULTS**

**Survival curve, intestinal bacterial load and splenic colonization by Salmonella Typhimurium**

No significant differences in survival time were observed between AL and CR mice after intragastric infection of 10⁴ CFU of Salmonella Typhimurium (Figure 1A; P>0.05, log-rank test), although there was a trend towards prolonged animal life in the CR group. Thus it is possible that a stronger restriction of diet would have a significant effect on the survival time of mice.

No clinical signs of salmonellosis were observed during the course of infection. The course of bacterial elimination in faeces from AL and CR mice following infection with 10⁴ CFU of Salmonella Typhimurium was evaluated for 14 days post-infection (Figure 1B). From day 1 to 14 post-infection, bacterial elimination in faeces was significantly lower in CR mice than in AL mice (P < 0.01), suggesting that the immune responses that control the proliferation of Salmonella Typhimurium in the small intestine were more effective in the CR group. A deficiency in the innate and adaptive immune response results in increased colonization of the spleen by the virulent strain of Salmonella Typhimurium [34]. Hence, we analysed the course of the systemic infection by quantifying CFU in the spleen, and found that this value was lower in CR mice than in AL mice, suggesting a superior immune response in the CR mice.

**EPR spectroscopy**

EPR studies were performed to determine the FR levels in liver and skeletal muscle. The EPR spectra from liver (Figures 2A–2F) seemed to consist of two independent features, the first with g = 2.04 and the second, an isotropic signal superimposed, with g = 2.008 and a linewidth of Γ = 1.1 mT.

The first signal (g = 2.04) is made up of several active species. By a regression-based spectral analysis, it was found that there are at least three paramagnetic species: haem iron–nitrosyl, DNIC (non-haem iron–nitrosyl) and ceruloplasmin.

The haem iron–nitrosyl signal has three principal g values (2.07, 2.00 and 1.98) and distinct three-line hyperfine splitting (αN = 1.6 mT) [35]. The DNIC signal turned out to be anisotropic and axial, with g⊥ = 2.04 and g∥ = 2.014 [19,33]. In Figure 2(G) the EPR signals of haem and non-haem iron–nitrosyl are similar to those previously reported [19,33]. However, the shape of the signal for the Hb–NO complex depends on the quaternary structure of Hb, which explains the apparent difference between the signal of Hb–NO (Figure 2G) and that of the tissue samples [35]. The existence of these species in control samples is due to endogenous NO production by l-arginine. In this sense, it has been proposed that a certain quantity of DNIC is formed...
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Figure 1 Survival after Salmonella Typhimurium infection, fecal excretion of the bacteria and colonization of spleen

After oral inoculation with $10^5$ CFU of serovar Typhimurium, the percentage of surviving mice (A) and the number of bacteria present in fecal pellets (B) were recorded daily during the 14 days post infection. The colonization of spleen was determined on days 7 and 14 (C). Results are expressed as means ± S.E.M. Whereas survival was similar in both CR mice and AL mice (log-rank test), the number of bacteria was significantly higher in AL mice after the first day post inoculation ($P < 0.01$, Student’s $t$ test). Colonization of spleen was higher in AL mice ($P < 0.001$, Holm–Sidak test).

endogenously in any organism capable of generating NO [36]. Furthermore, this signal can be assigned to ceruloplasmin, which has a broad isotropic signal with $g = 2.05$ and has been found in samples from blood, liver and kidney [29,37]. However, the contribution of this protein at this signal could be contradictory, because ceruloplasmin levels are increased during infection but decreased with CR [38]. Therefore the detection of any single signal in the sample could be difficult due to overlapping.

The second signal ($g = 2.008$), which was found in liver and skeletal muscle samples, was assigned to the presence of non-specific delocalized unpaired electrons. In biological samples various amounts of this kind of electron are present, being produced by normal cell metabolism [39,40] from components of the mitochondrial respiratory chain, such as flavosemiquinones. The latter have been reported in the EPR spectra of normal liver [41].

The signals detected by EPR, at both $g = 2.04$ and $g = 2.008$, were different between liver and skeletal muscle samples, which can be explained by the fact that each organ has different physiological functions and therefore distinct types and quantities of proteins. Examples of enzymes that can be found in different quantities are those from the mitochondrial electron transfer, such as cytochromes, ubiquinone reductase and aconitase [37].

According to the area under the EPR signal, which is directly proportional to the quantity of FRs present in the sample [42], among the uninfected control groups the AL mice (Figure 2B) had significantly greater FR levels in liver samples than did the CR (Figure 2A) mice (Figure 2H; $P < 0.05$). In contrast, among the infected groups, at 7 days post-infection CR mice (Figure 2C) had significantly greater FR levels than did AL (Figure 2D) mice (Figure 2H; $P < 0.05$).

Comparing, the uninfected CR mice with the CR mice that were infected for 7 days, the FR levels in the infected mice, while higher, did not reach statistical significance. While the FR levels were greater in CR mice infected for 7 as compared with 14 days (Figure 2E), as evidenced by the area under the curve of the representative signal at $g = 2.008$ for liver samples (Figure 2H), the difference in FR levels between AL mice infected for 7 (Figure 2D) as compared with 14 days was not significant (Figure F).

The EPR spectra of skeletal muscle samples from uninfected CR (Figure 3A) and AL (Figure 3B) mice showed a single signal at $g = 2.008$, which had a significantly lower intensity for CR than for AL mice, confirming that FR production was lower in healthy mice with a CR diet. However, 7 days post infection, the intensity of this signal was significantly higher in CR (Figure 3C) than in AL (Figure 3D) mice (Figure 2G; $P < 0.05$). Nevertheless, at 14 days post infection, no significant difference was observed between CR (Figure 3E) and AL (Figure 3F) mice in this respect.

Nitrate/nitrite determination

NO quantification was carried out by the determining nitrite, the product of NO oxidation, levels. L-Arginine, which was present in the diet used in the present study, can lead to higher NO production [43]. However,

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Figure 2  EPR spectra and EPR signal area of liver samples

Representative EPR spectra of liver samples: from uninfected mice provided with a CR (A) or AL (B) diet, at 7 days post infection with *Salmonella* Typhimurium from mice provided with a CR (C) or AL (D) diet, and at 14 days post infection from mice provided with a CR (E) or AL (F) diet. The EPR spectra of DNIC (continuous line) and the Hb–NO complex (dotted line) are shown (G). The areas under the EPR signal at $g = 2.008$ (the mean ± S.E.M.), representing the average for at least three independent experiments (H). $P < 0.05$ was accepted as statistically significant, evaluated by one-way ANOVA followed by the Holm–Sidak test.

In the present study, a much smaller amount of L-arginine was contained in the diet than in the aforementioned study, and our results show that, in uninfected mice, there was no significant difference in the content of nitrites between the liver samples from AL and CR mice (Figure 4A), despite the fact that the animals fed AL consumed more L-arginine. Therefore the L-arginine contained in the diet, apart from being used in NO production, was probably also employed in another metabolic function.

In contrast, a significant difference ($P < 0.05$) was found at 7 days post infection between the CR and AL mice, with the former having greater amounts of nitrites. The level of nitrites in the AL mice was so low that it is not observable on the graph. If the quantity of nitrites in the animals depended on diet, greater amounts would have been detected in the AL groups. The fact that a greater amount was actually found at 7 days post infection in the CR than in the AL mice suggests that the quantity of nitrites was determined by the immunological response rather than by the diet. There was no significant difference between the CR and AL mice in nitrite levels at 14 days post infection, possibly due to a lower immune response by this time, which would involve less NO production.

Figure 4(B) shows that the samples of skeletal muscle from uninfected mice had significantly higher amounts of nitrites in CR than AL mice. These results are in accordance with previous published reports [44]. However, in the samples from infected mice, the nitrite levels of the AL and CR groups were not significantly different, although there was a trend towards lower nitrite levels in the latter.

**iNOS expression**

The EPR results and nitrite determination indicate that the samples from CR mice had higher amounts of NO than those of AL mice after being infected, which may be associated with a better immune response, principally in liver. The CR mice infected for 7 or 14 days showed higher expression of mRNA of NOSII in liver samples ($\sim$50-fold and $\sim$17-fold respectively) compared with the uninfected CR animals (Figure 5). In addition, whereas infected CR mice had more NOSII mRNA than infected AL animals at 7 days post infection, by day 14 post infection there was no significant difference between the two groups. A significant difference was observed in this value between the liver samples of the infected AL mice at 14 days post infection and the uninfected AL animals. The high expression of NOSII in infected CR animals is in accordance with their high nitrite level, as it
has been reported that NOSII produces an approx. 5-fold higher amount of NO than constitutive NOS isoforms [45].

SOD activity

SOD is the enzyme responsible for converting the superoxide anion to hydrogen peroxide. The superoxide anion interacts with nitric oxide to produce peroxynitrite (ONOO⁻), a consequence of the fact that peroxynitrite production is faster than hydrogen peroxide production [46,47]. The catalytic activity of SOD could have been modified due to a lesser availability of its substrate. SOD activity analysis of liver (Figure 6A) and skeletal muscle (Figure 6B) samples from uninfected mice show that there was no significant difference between the AL and CR groups (Figure 6). However, at 7 days post-infection the SOD activity decreased in both tissues, independently of whether the mice were fed AL or with the CR diet.

It has been reported that NOSII can produce O₂⁻• depending on substrate and cofactor availability [45]. However, despite the fact that CR animals had less food, no difference was observed between their SOD activity and that of the AL group.

DISCUSSION

The main findings of the present work are that: (i) non-infected mice produced fewer FRs while on a CR diet compared with an AL diet; (ii) infected mice produced more FRs, expressed more NOSII mRNA and produced more NO in the liver at 7 days post infection while on a CR diet compared with an AL diet; (iii) there was less colonization of the spleen after oral infection with Salmonella Typhimurium in CR mice compared with AL mice; and (iv) CR mice cleared the infection more quickly than AL mice.

Because few studies have used an intact bacteria inoculation to test whether the enhanced capacity of the immune response during CR can modify the susceptibility of hosts to intact pathogens [11], we compared the susceptibility of CR and AL mice with oral infection with Salmonella Typhimurium, an intracellular pathogen eliminated by innate and adaptive immunity.

Although oral doses of 10⁶ CFU of Salmonella Typhimurium did not cause clinical symptoms of salmonellosis, the intestinal lumen and spleen were colonized by the bacteria in AL and
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Figure 4 Nitrite content in samples from liver (A) and skeletal muscle (B) of uninfected mice and animals infected with *Salmonella* Typhimurium with a CR or AL diet

P < 0.05 was accepted as statistically significant, evaluated by one-way ANOVA followed by the Holm–Sidak test.

CR mice, and there were differences in the course of infection between the two groups. The number of *Salmonella* Typhimurium cells in the intestinal lumen and spleen was lower in CR than AL mice, indicating that CR increased resistance to infection. Our results are in agreement with a previous study that demonstrated that long-term CR improved resistance in mice injected intraperitoneally with *Salmonella* Typhimurium [10].

In healthy animals, there was a lower production of FRs in CR mice compared with AL mice, in agreement with the reports of diminished ROS production in uninfected animals on a CR diet [48–51]. On the other hand, during the acute phase of infection with *Salmonella* Typhimurium, a higher production of FRs was found in liver samples from CR mice compared with AL mice. FRs probably contributed to the rapid elimination and control of *Salmonella* Typhimurium infection in CR mice, given that innate immune responses are the first line of defence against an invasion by this pathogen. This innate response includes phagocytosis and intracellular killing of *Salmonella* Typhimurium by phagocytic cells (macrophages and neutrophils) [34]. To the best of our knowledge, the effect of a CR diet on the production of FRs in mice infected with *Salmonella* Typhimurium has not yet been analysed.

NO apparently plays a relevant role in host resistance to *Salmonella* Typhimurium infection [19,52]. Although it is difficult to directly measure this molecule, as it has a very short *t* 1/2 and diffuses through all biological barriers [53], the iron–nitrosyl complex, a metabolic product of NO, can be easily measured and may be a useful correlate of NO production [54,55]. In biological samples, NO is a cytotoxic molecule that causes enzymatic inhibition by forming complexes with haem or non-haem iron, complexes which have different EPR signals. Whereas the haem iron–nitrosyl complex exhibits a rhombic symmetry and a usually resolved nitrogen (14N) hyperfine triplet structure, the non-haem iron–nitrosyl complex shows an axial signal [56]. NO production can be detected by both Hb–NO and non-haem iron–nitrosyl complexes. Therefore the change in the area under these EPR signals in animals infected with *Salmonella* Typhimurium is due to the cytotoxic activity of macrophages [56]. Such cytotoxic activity is characterized by a loss of intracellular iron and an inhibition of certain enzymes that have a catalytically active non-haem-iron atom co-ordinated to a sulfur group, as the iron–sulfur groups are sensitive targets for iron–nitrosyl complex formation [56].

In the present study, a stronger EPR signal was detected in the infected mice on a CR diet compared with an AL diet, which suggests that CR provides the conditions for greater resistance against *Salmonella* Typhimurium infection. The nitrite concentration was higher in liver samples from infected CR mice compared with infected AL mice at 7 days post infection, confirming higher NO production in the former group. Furthermore, NOSII expression (detected by mRNA) was greater in infected...
Figure 6 SOD activity in liver (A) and skeletal muscle (B)

ALC, CRC, ALS (ad libitum infected with Salmonella Typhimurium for 7 or 14 days), CRS (CR, infected with Salmonella Typhimurium for 7 or 14 days). Results represent means ± S.E.M. for three animals. P < 0.05 was accepted as statistically significant, evaluated by one-way ANOVA followed by the Holm–Sidak test. U, units.

CR mice than in infected AL mice. It was also greater in infected than in uninfected animals, which is in accordance with previous reports demonstrating that NOSII is induced after Salmonella Typhimurium infection [20,52]. These results corroborate that CR improves innate and adaptive immune responses, including a greater production of NO by activated macrophages and other liver cells [57–59]. These three parameters, the iron–nitrosyl signal, nitrite concentration and NOSII expression, were higher in samples from animals at 7 days post-infection compared to 14 days post infection, indicating that this response is time-dependent.

Because hepatocytes and non-parenchymal cells (endothelial cells, Kupffer cells and stellate cells) produce NO via NOSII induction, the production of an iron–nitrosyl complex is probably a result of the in vivo activation of those cells by bacterial products such as lipopolysaccharide [60–62]. An additional source of NO is the bacteria itself, Salmonella Typhimurium grown anaerobically is capable of generating NO by the activity of a membrane-bound nitrate reductase. However, this source of NO probably is not operating in vivo because under aerobic conditions, as inside the phagolysosome, a functional bacterial flavohaemoglobin detoxifies the NO produced by Salmonella Typhimurium [63].

The fact that in healthy mice FR production was lower in CR mice compared with AL mice, but higher in infected mice on a CR diet compared with those on an AL diet, is probably related to a different production of ROS and RNS by activated macrophages in infected mice compared with uninfected CR and AL mice. IFN-γ (interferon-γ) and TNF-α (tumour necrosis factor-α) are essential during the initial stages of Salmonella Typhimurium infection because they are involved in the induction of bactericidal mechanisms (e.g. FR production) of macrophages [34]. Recently, we found that CR increases the production IFN-γ and TNF-α by macrophages and parenchymal cells of the liver [64]. These cells probably produce more FRs, which can contribute to the elimination of the pathogen [9,12,61]. This was the case in the present study, as evidenced by higher expression of NOSII and greater production of NO in liver of infected mice with a CR than with an AL diet.

Conclusions
In response to Salmonella Typhimurium infection, a better defence mechanism was found in CR mice compared with AL mice. Among infected mice there was a higher NOSII expression, a greater production of the iron–nitrosyl complex, and a higher nitrite production in mice provided with a CR compared with an AL diet. All these parameters indicate increased NO production caused by the activation of macrophages, which could explain the more effective immune response observed in CR infected mice.

AUTHOR CONTRIBUTION
Rafael Campos-Rodríguez and Eleazar Lara-Padilla undertook the treatment of the animals and splenic colonization. Daniel Ramírez-Rosales and Rafael Zamorano Ulloa performed the EPR spectroscopy experiments. José Trujillo-Ferrara, José Correa Basurto and Martha C. Rosales-Hernández were involved in animal handling and sample isolation, nitrate/nitrite determination, SOD activity and the EPR spectroscopy experiments. Ángel Miliar García and Humberto Reyna Garfias performed the RNA extraction and cDNA synthesis, and the quantitative real-time PCR assay. All authors contributed in the interpretation, analysis and discussion of the results, as well as in the writing of the paper.

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