Attenuation of post-myocardial infarction depression in rats by \( n-3 \) fatty acids or probiotics starting after the onset of reperfusion

Kim Gilbert\(^1,2\), Jessica Arseneault-Bréard\(^1,2\), Fabio Flores Monaco\(^1\), Alexanne Beaudoin\(^1\), Thierno Madjou Bah\(^1,3\), Thomas A. Tompkins\(^4\), Roger Godbout\(^1,3\) and Guy Rousseau\(^1,2\)*

\(^1\)Centre de biomédecine, Hôpital du Sacré-Cœur de Montréal, 5400 Boulevard Gouin Ouest, Montréal, Québec, Canada H4J 1C5
\(^2\)Département de pharmacologie, Université de Montréal, CP Centre-Ville, Montréal, Québec, Canada H3C3J7
\(^3\)Département de psychiatrie, Université de Montréal, CP Centre-Ville, Montréal, Québec, Canada H3C3J7
\(^4\)Institut Rosell-Lallemand Inc., 6100 Royalmount, Montréal, Québec, Canada H4P 2R2

(Submitted 30 March 2012 – Final revision received 26 July 2012 – Accepted 26 July 2012 – First published online 15 October 2012)

Abstract
Proinflammatory cytokines play a central role in depression-like behaviour and apoptosis in the limbic system after myocardial infarction (MI). A PUFA \( n-3 \) diet or the combination of \textit{Lactobacillus helveticus} R0052 and \textit{Bifidobacterium longum} R0175 probiotics, when given before the ischaemic period, reduce circulating proinflammatory cytokines as well as apoptosis in the limbic system. The present study was designed to determine if the same nutritional interventions maintain their beneficial effects when started after the onset of the reperfusion period and attenuate depression-like behaviour observed after MI. MI was induced by the occlusion of the left anterior descending coronary artery for 40 min in rats. After the onset of reperfusion, animals were fed with a high- or low-PUFA \( n-3 \) diet, combined or not with one billion live bacteria of \textit{L. helveticus} and \textit{B. longum}. At 3 d post-MI, caspase-3 enzymatic activities and terminal 2'-deoxyuridine, 5'-triphosphate (dUTP) nick-end labelling (TUNEL)-positive cells were decreased in the CA1, dentate gyrus (DG) and amygdala with the high-PUFA \( n-3 \) diet, as compared to the three other diets. Probiotics attenuated caspase-3 activity and TUNEL-positive cells in the DG and the medial amygdala. At 2 weeks post-MI, depression-like behaviour was observed in the low-PUFA \( n-3 \) diet without probiotics-group, and this behaviour was attenuated with the high-PUFA \( n-3 \) diet or/and probiotics. These results indicate that a high-PUFA \( n-3 \) diet or the administration of probiotics, starting after the onset of reperfusion, are beneficial to attenuate apoptosis in the limbic system and post-MI depression in the rat.

Key words: \( n-3 \) Fatty acids; Apoptosis; Limbic system; Diet; Behaviour; Hippocampus; Amygdala

Depressive mood is common after myocardial infarction (MI)\(^1\) and 20\% of MI patients develop an episode of major depression\(^2\). The association of depression with MI is critical, as the mortality rate is increased three to four times in depressed MI patients\(^3\). As the level of non-responders is still high with depressed MI patients compared to non-depressed MI patients\(^3\), the physiopathology of depression is complex and different mechanisms are involved\(^7\). Our previous work and that of others indicate that proinflammatory cytokines may play a key role in depression-like behaviour\(^8\)–\(^10\). Indeed, injection of proinflammatory cytokines is followed by depression-like behaviour\(^11\). Post-MI increased levels of proinflammatory cytokines are accompanied by apoptosis in the hippocampus and the amygdala, another physiopathological element of depression\(^8,9,12\).

Dietary interventions can be used to prevent the post-MI elevation of proinflammatory cytokines. For example, we have shown that high-PUFA \( n-3 \) diets reduce the circulating level of TNF\(\alpha\) after MI\(^{13}\). High-PUFA \( n-3 \) diets also reduce levels of monocyte chemotactic protein-1 (MCP-1), a chemokine that contributes to inflammatory states\(^{14,15}\). In parallel, it has been observed that \textit{Lactobacillus helveticus} R0052 reduces proinflammatory cytokines IL-1\(\beta\) and IL-6, but nonsignificantly reduces TNF-\(\alpha\)\(^{16}\), whereas \textit{Bifidobacterium longum} R0175 can reduce TNF-\(\alpha\) as well as IL-8\(^{17}\). Combined

Abbreviations: AR, area at risk; CA1, hippocampal CA1; CA3, hippocampal CA3; DG, dentate gyrus; LA, lateral amygdala; MA, medial amygdala; MCP-1, monocyte chemotactic protein-1; MI, myocardial infarction; TUNEL, terminal dUTP nick-end labelling.

* Corresponding author: Dr G. Rousseau, fax +1 514 338 2694, email guy.rousseau@umontreal.ca
Experimental methods

Ethics statement

These experiments conformed to animal care guidelines published by the Canadian Council on Animal Care, and the procedures performed were approved by the local Animal Care Committee of Hôpital du Sacré-Coeur de Montréal.

Experimental design

We studied a total of sixty-four male, 3-month-old Sprague–Dawley rats weighing 325–350 g at the start of the experiments (Charles River Canada). Rats were housed individually under constant conditions (temperature of 21–22°C and humidity of 40–50%), including a 12 h dark–12 h light cycle, which began at 08:00 hours. Chow pellets and tap water were available ad libitum throughout the study. An acclimatization period of 3 d after delivery by the supplier was allowed before the rats were randomly distributed in one of the four groups: a low- or high- n-3 PUFA diet in combination or not with probiotics L. helveticus R0052 and B. longum R0175 (mentioned later). Six rats from each group (a total of twenty-four) were killed 72 h post-MI for biochemical analyses before heart ischaemia, the present protocol aimed at implementing the nutritional intervention after MI in order to increase clinical relevance.

Diets

Chow pellets rich in lipid (22% protein, 42.9% carbohydrate, 20.3% fat, by weight) were supplied by Harlan Teklad. The respective composition in fatty acids is presented in Table 1.

Probiotics

The probiotics that were given were a commercially available combination of two genera: L. helveticus R0052 and B. longum R0175 (Probio'Stick™ provided by Institut Rosell, Inc.). The probiotics were administered by dissolving the freeze-dried culture or the vehicle only (maltodextrin) in 200 ml of drinking tap water. Each rat in the probiotics group received a daily dose of 10^9 colony-forming units. The drinking solution was newly prepared every 2nd day for the duration of experiments. Water intake was monitored throughout the entire investigation to ensure that sufficient bacteria were administered. The body weight of each rat was also monitored every day.

Surgical procedure

Animals were anaesthetised following a ketamine/xylazine (50 and 5 mg/kg, respectively) intramuscular injection. Subsequently, the rats were intubated and anaesthesia was maintained using isoflurane (1.2%) ventilation. Electrocardiogram and heart rate of the rats were monitored throughout the procedure using electrodes placed on their paws. A left thoracotomy was carried out at the fifth intercostal space, permitting the occlusion of the left anterior descending coronary artery using a 4–0 silk suture (Suturex; Coviidien) and plastic snare. Ischaemia was confirmed by alterations of the ST segment and the presence of the ventricular subepicardial cyanosis. After 40 min of ischaemia, the thread was removed, permitting reperfusion of the myocardial tissue. Then, the thorax was sutured by means of a 2–0 silk suture (Suturex; Covidien) and the animals were given an antibiotic intramuscular injection (15 000 U penicillin G; Duplocillin LA, Intervet Canada Limited) as well as an analgesic subcutaneous injection (2 mg/kg buprenorphine) before being returned to their respective cages. The animals received a second analgesic injection (0.05 mg/kg buprenorphine) the day after surgery.

Table 1. Composition of PUFA in the different diets (g/kg)

<table>
<thead>
<tr>
<th>Diets</th>
<th>High</th>
<th>Low</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total fat</td>
<td>202.5</td>
<td>202.5</td>
</tr>
<tr>
<td>Saturated fat</td>
<td>78.1</td>
<td>71.6</td>
</tr>
<tr>
<td>Monounsaturated fat</td>
<td>33.7</td>
<td>34.8</td>
</tr>
<tr>
<td>Polysaturated fat</td>
<td>83.2</td>
<td>92.5</td>
</tr>
<tr>
<td>n-6 PUFA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18 : 2n-6 (LA)</td>
<td>38.9</td>
<td>76.7</td>
</tr>
<tr>
<td>20 : 4n-6 (AA)</td>
<td>1.3</td>
<td>0</td>
</tr>
<tr>
<td>n-3 PUFA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18 : 3n-3 (ALA)</td>
<td>22</td>
<td>15.6</td>
</tr>
<tr>
<td>20 : 5n-3 (EPA)</td>
<td>9.3</td>
<td>0</td>
</tr>
<tr>
<td>22 : 5n-3 (DPA)</td>
<td>2.3</td>
<td>0</td>
</tr>
<tr>
<td>22 : 6n-3 (DHA)</td>
<td>6.3</td>
<td>0</td>
</tr>
</tbody>
</table>

n-3 Fatty acids, probiotics and depression
The hearts were removed and washed with saline by retrograde perfusion into aorta. The left anterior descending coronary artery was occluded at the same site as for inducing MI (as mentioned earlier) to map the area at risk (AR) using an infusion of Evans blue (0·5 %). The hearts were frozen (−80°C for 5 min), sliced into four transverse sections of 2 mm and placed in 2,3,5-triphenyltetrazolium (1 %, pH 7·4) at 37°C for 10 min to better distinguish the area of necrosis (I) from AR. The MI was expressed as a percentage of necrosis (I) of the AR ((I/AR) × 100). Additionally, AR was expressed as a percentage of left ventricle area (LV) ((AR/LV) × 100).

Biochemical analysis

Caspase-3 activity. Caspase-3 activity was measured according to the protocol described previously (19). Tissues were homogenised by sonification in lysis buffer and incubated for 30 min on ice. The tissue homogenates were centrifuged at 4°C for 10 min. Enzymatic reactions were undertaken in reaction buffer with 25 mg of protein (attested by the Bradford method) and fluorescent substrate (Ac-DEVD-AMC; CEDAR-LANE; 40 μM). Reactions were studied after incubation in the dark for 3 h at 37°C, and stopped with the addition of 0·4 M-NaOH and 0·4 M-glycine buffer. Fluorescence was quantified by spectrofluorometry (Photon Technology International) at an excitation wavelength of 365 nm and emission wavelength of 465 nm.

Terminal dUTP nick-end labelling-positive cells

TUNEL analysis was undertaken according to the protocol provided by the manufacturer (Oncogene Science, Inc.) and according to the TUNEL protocol described previously (8). In summary, cryostat sections were fixed in paraformaldehyde (4 %) and rinsed, then permeabilised and, after washing, the labelling reaction was performed for 1 h at 37°C. The reaction was stopped, and all nuclei were stained with propidium iodide (1 μM). The sample was viewed in a Leica epifluorescence microscope with a filter for 4′,6-diamidino-2-phenylindole (DAPI) (330–380 nm). All sections were photographed at the same magnification with a Nikon Coolpix 995 camera (Nikon Canada). A total of 20–25 pictures were taken of each sample, with quantification of positive cells on total number.

Rat cytokines/chemokines assay

A cytokine/chemokine assay was performed according to the protocol provided by the manufacturer (Rat Cytokine/Chemokine Magnetic Bead 96-Well Plate, Millipore Canada). In brief, the plasma was diluted by 5-fold and 200 μl of assay buffer was added into each well of the plate, mixed for 10 min at room temperature and decanted. The diluted samples were incubated overnight at 4°C in the reaction mix in the presence of beads, and the next morning, the plates were washed and incubated for 2 h at room temperature with detection antibodies. After that, streptavidin–phycoerythrin solution was added to the wells for 30 min followed by the addition of sheath fluid. Plates were run on a Bio-Plex 200 system with high-throughput fluidics (Bio-Rad). The median fluorescent intensity was analysed to determine the plasma concentrations of MCP-1 and IL-4.

Behavioural measures

The tests employed were selected on the basis of their validity regarding behavioural depression syndrome. All tests were conducted individually, in the morning, starting 12 d after surgery. Social interaction was evaluated on day 12, the forced swimming test was performed on days 15 and 16 and the passive avoidance step-down test on day 17. All animals were killed on day 18.

Social interaction test

Pairs of rats were each placed in a clean shoebox for 10 min. During this period, two observers without knowledge of the experimental condition observed one animal each; the duration and number of interactions with the other rat was measured. Rats were tested between 09.00 and 11.00 hours.

Forced swim test

Rats were placed individually in a transparent 25 cm diameter pool filled to a depth of 30 cm with 22–25°C water, with no possible escape. Two observers without knowledge of the experimental condition used identical chronometers to time the immobile, swim and escape trial periods of the animals. The test was conducted for 2 d: day 1 comprised 15 min of habituation and day 2 entailed the actual 5 min test. On the 2nd day, immobility time was translated into depressive symptoms by comparison to the controls. Rats were tested between 09.00 and 11.00 hours.

Passive avoidance step-down test

Rats were placed individually in a test chamber (14 cm × 23 cm) on a Plexiglas platform (14 cm × 19 cm). An electrifiable grid (14 × 14 cm) is placed alongside and 2·5 cm lower than the Plexiglas platform (14 cm × 14 cm). When the
animal places all four feet on the electrifiable grid, it receives a mild, brief shock (5 mA for 1 s) and is removed from the test chamber. After 30 s, it is placed anew on the platform. If the rat remains on the platform without going onto the grid for 1 min, it is removed from the test box for 30 s. Criterion is reached when the rat avoids going onto the grid for three consecutive trials. The number of trials needed to reach the test criterion and the time needed to learn the test were noted. Rats were tested 1 d before they were killed.

Statistical analysis

Data are reported as means with their standard errors. Groups were compared with a two-way factorial ANOVA, using diet and probiotics as factors. If an interaction between diet and probiotics was significant, simple effects were analysed according to Field(20), otherwise if an interaction was not significant, main effects were reported. The MI size and AR were compared with a two-way factorial ANOVA, using diet and probiotics as factors. If an interaction between diet and probiotics was significant, simple effects were analysed according to Field(20).

Results

Food, water consumption and weight gain were similar between groups throughout the protocol (data not shown), indicating that diet had no influence on these measures.

Infarct size

After 72 h and 18 d post-MI reperfusion, MI size was similar between groups and represents about 35% of the AR (data not shown). This indicates that diets had no protective effect on the ischaemic myocardium when started during the reperfusion period.

Apoptosis

No significant interaction was observed for caspase-3 activity among the five regions tested after 72 h of reperfusion. Main effect analysis indicated that caspase-3 activity was significantly higher in the low- n-3 PUFA diet compared to the high- n-3 PUFA diet (diet factor) in the CA1 (F(1,16) = 24.04; P<0.05), DG (F(1,19) = 11.27; P<0.05) and in the LA (F(1,15) = 11.77; P<0.05) (Fig. 1). Caspase-3 activity was similar in the CA3 and MA for the diet factor (Fig. 1). Analyses of the probiotic factor indicated a significant reduction in caspase-3 activity in the DG (F(1,19) = 4.68; P<0.05) and MA (F(1,15) = 5.63; P<0.05) areas. No significant difference was observed in the CA1, CA3 and in the LA for the probiotic factor (P>0.05; Fig. 1).

The TUNEL-positive cells were significantly increased in the low- n-3 PUFA diet compared to the high- n-3 PUFA diet in the CA1, DG, LA and MA regions. The probiotics group showed a decrease in the TUNEL-positive cells in the DG and MA regions v. the placebo group (P<0.05; Table 2). No other difference was observed.

Behavioural tests

Analyses of social interaction duration disclosed a significant interaction between diet and probiotics (F(1,28) = 5.34; P<0.05). Analysis of simple effects revealed lower values in the low- n-3 PUFA diet without probiotics compared to the low- n-3 PUFA with probiotics (F(1,29) = 7.40; P<0.05), indicating the beneficial effect of probiotics in the presence of the low- n-3 PUFA diet (Fig. 2).

Fig. 2. Social interaction – results of social interaction test proceed on the 12th day of reperfusion in seconds. Values are means, with their standard errors represented by vertical bars (n 8–10 per group). * Indicates a significant interaction (P<0.05).

Table 2. Percentage of terminal dUTP nick-end labelling-positive cells on total cell count in different limbic regions analysed at 72 h after reperfusion

<table>
<thead>
<tr>
<th></th>
<th>High-PUFA n-3 diet</th>
<th>Low-PUFA n-3 diet</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without probiotics</td>
<td>With probiotics</td>
<td></td>
</tr>
<tr>
<td>CA1</td>
<td>1.40</td>
<td>1.23</td>
<td></td>
</tr>
<tr>
<td>CA3</td>
<td>0.58</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>DG</td>
<td>1.45</td>
<td>1.05</td>
<td></td>
</tr>
<tr>
<td>LA</td>
<td>0.98</td>
<td>0.92</td>
<td></td>
</tr>
<tr>
<td>MA</td>
<td>1.87</td>
<td>1.11</td>
<td></td>
</tr>
</tbody>
</table>

CA1, hippocampal CA1; CA3, hippocampal CA3; DG, dentate gyrus; LA, lateral amygdala; MA, medial amygdala.

CA1, hippocampal CA1; CA3, hippocampal CA3; DG, dentate gyrus; LA, lateral amygdala; MA, medial amygdala.
trials indicated a significant difference between groups ($\chi^2 = 7.89$, df = 3, $P<0.05$). The results indicate that the low- $n$-3 PUFA diet without probiotics group took more attempts to succeed the test, as compared to the low- $n$-3 PUFA diet with probiotics group ($P=0.029$).

**Cytokine/chemokine assay**

The probiotic diet was associated with significantly higher IL-4 plasma concentrations compared to vehicle ($F(1,24) = 6.65; P<0.05$). The low- PUFA $n$-3 diet was associated with significantly higher MCP-1 plasma concentrations compared to the high-PUFA $n$-3 diet ($F(1,30) = 4.74; P<0.05$; Fig. 5).

**Discussion**

The data obtained in the present study show that a high- $n$-3 PUFA diet implemented for 2 weeks after a MI reduces depression-like behaviour, as documented by three different behavioural tests. Similarly, the combined intake of probiotics *L. helveticus* R0052 and *B. longum* R0175 had a positive effect on depression-like behaviour in presence of a low- $n$-3 PUFA diet. The addition of probiotics to the high- $n$-3 PUFA diet has no additional effect on behaviour after MI, suggesting a common pathway of action between $n$-3 and probiotics, or alternatively, a plateau effect on behaviour is reached with the high- $n$-3 PUFA diet without any possibility of improvement in the presence of probiotics.

Both nutritional interventions (3- and probiotics) were started after the onset of reperfusion and did not have a significant effect on infarct size, as expected. Indeed, cardioprotective interventions need to be started at the onset of reperfusion or soon after to be beneficial for the myocardium. We have shown, for example, that the administration
of an A2a adenosine receptor agonist, CGS21680. 5 min before the reperfusion period reduced infarct size, whereas its administration 5 min after the onset of reperfusion did not. In the present study, n-3 or probiotics were started after the animals recovered from anaesthesia, i.e. a few hours after the onset of reperfusion.

In previous studies, we documented the presence of apoptosis in the amygdala and hippocampus following MI, with a peak at about the 3rd day of reperfusion. Interestingly, pharmacological interventions with antidepressants (sertraline) or others (probiotics) have been shown to reduce apoptosis and also to attenuate the depression-like behaviour. The increase in cell death perturbs the homeostasis of the limbic system and can be responsible for the depression-like behaviour. Indeed, it has been shown that neurons that have neither electrical activity nor trophic support die by apoptosis and also to attenuate the depression-like behaviour. The difference between the n-3 diets can be explained by the presence of EPA and DHA (long-chain PUFA) in the high-n-3 diets and their absence in the low-n-3 diet. EPA and DHA are beneficial for the heart and the brain and it has been observed that elongation of α-linolenic acid, although limited, can occur in rats and produce DHA and EPA. In the low-n-3 diet, however, α-linolenic acid levels may be insufficient or the delay to produce DHA and EPA may be too long to have a positive effect.

**Conclusion**

In conclusion, the present data indicate that a high-n-3 PUFA diet or addition of *L. helveticus* R0052 and *B. longum* R0175 to a low-n-3 PUFA diet is beneficial after MI to reduce apoptosis in the limbic system and to attenuate depression-like behaviour.

**Acknowledgements**

The present work was supported by La Fondation des Maladies du Coeur du Québec. The authors declare no conflict of interest. The authors want to thank Institut Rosell Lallemand for providing the probiotics (*L. helveticus* R0052 and *B. longum* R0175) and the vehicle. K. G., J. A.-B., F. F. M., A. B. and T. A. T. contributed to the experiments, data analysis and writing, and R. G. and G. R. contributed to the conception of the experiments, data analysis and writing.