SCIENTIFIC DOSSIER

Lactium®

Stress management through nutrition

TABLE OF CONTENTS

In the past decades, a large number of milk-derived peptides with bioactive properties have been discovered. They are encrypted in the sequence of the parent protein and may be released in the gastrointestinal tract by enzymatic digestion (Hartmann & Meisel 2007). The first enzymes responsible for protein digestion are pepsin in the stomach followed by trypsin and chymotrypsin in the small intestine. Thereafter specific proteases continue the breakdown to di- and tri-peptides as well as amino-acids which can subsequently be absorbed.

The observation of the calm state of babies after feeding led to the hypotheses that either the milk contains a soothing substance or the babies' digestive tract is able to release such a substance from the ingested milk. A substance with those properties would most probably be a peptide derived from protein hydrolysis.

Based on these hypotheses, the research team of Prof. Linden at the Henri Poincaré University in Nancy, France (today University of Lorraine) discovered a tryptic hydrolysate from bovine α_{S1}-casein that displayed anxiolytic-like properties in two classical animal models of anxiety (Miclo *et al.*, 2001).

α-casozepine

Of all peptides present in the hydrolysate with anxiolytic properties, one displayed an affinity to the binding site of benzodiazepine on the central $GABA_A$ receptor, a class of drugs which have amongst others anxiolytic, hypnotic and sedative properties (Miclo *et al.*, 2001). This affinity was however much lower than the one of benzodiazepine. No affinity was observed for the peripheral binding site of benzodiazepine. Today the peptide identified in the original hydrolysate is known as α-casozepine.

The amino-acid sequence of α-casozepine was subsequently determined. α-casozepine corresponds to the sequence 91-100 of bovine α_{S1}-casein and consists of the amino acid sequence YLGYLEQLLR. Its anxiolytic activity was confirmed in further studies both with the purified and the synthesized form of the peptide (Miclo *et al.*, 2001).

In order to better understand its affinity with the benzodiazepine binding site of the $GABA_A$ receptor, the structure of α-casozepine was further studied (*Fig. 1*). Indeed, based on its three-dimensional conformation under physiological conditions, a fairly similar structure to benzodiazepine was found (Lecouvey *et al.*, 1997a & 1997b).

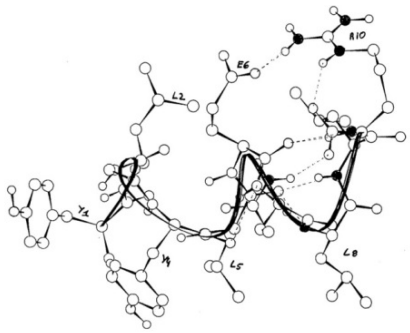


Figure 1 : Average structure of α-casozepine. Letters correspond to the amino acid sequence.

Lactium® - a hydrolysate of α_{S1}-casein

Subsequently to this work, INGREDIA developed a unique process to produce a specific hydrolysate containing α-casozepine on an industrial level.

Based on the original research, Lactium® is a tryptic hydrolysate from α_{S1}-casein. Its anxiolytic activity was validated and repeatedly confirmed in numerous studies conducted in different animal species, amongst others in rat, cat and dog (Beata *et al.*, 2007a, Beata *et al.*, 2007b). Clinical trials with stressed but otherwise healthy human volunteers were able to demonstrate relaxing and anti-stress properties of Lactium®.

Lactium® is safe for use as demonstrated in the toxicological assessment and does not show the adverse effects that are observed for benzodiazepine such as addiction, memory impairment, tolerance and disinhibition.

Data on these different studies can be found in the present scientific dossier.

Efficacy of Lactium® on stress and anxiety evaluated in preclinical studies

The goal of the present studies was to examine the anxiolytic effect of α-casozepine and Lactium® in two models of anxiety, the conditioned defensive burying (CDB) test and the Elevated Plus Maze (EPM) using male Wistar rats. These tests are respectively described in detail in appendices 1 and 2.

The rats used in the present studies were treated according to the rules provided by the ASAB (Association for the Study of Animal Behaviour) Ethical Committee (Guidelines for the use of animals in research, 1993) and by the Canadian Council on Animal Care (1984).

The following studies were carried out at:

ETAP-LAB
13 rue du Bois de la Champelle
54500 Vandoeuvre-lès-Nancy (France)

Dose response function for the anxiolytic-like effect of α-casozepine

Test aim

Using the CDB test, the study aimed at determining the effective dose for the anxiolytic-like effect of α-casozepine when administered intraperitoneally (i.p.).

Treatment

α-casozepine at doses of 0.2, 0.4 and 0.6 mg/kg body weight (BW) was dissolved in 0.9% NaCl solution. It was administered intraperitoneally 30 minutes before the test started. Control animals received the same volume of NaCl solution (negative control). Diazepam (1.0 mg/kg body weight) was used under the same conditions as a positive control for the model.

Results

At doses of 0.4 and 0.6 mg/kg body weight, α-casozepine significantly decreased the global anxiety score (GAS) in comparison with the negative control (*Fig. 2*). At 0.2 mg/kg body weight, a tendency for a decrease was observed. The positive control, diazepam, significantly decreased the global anxiety score.

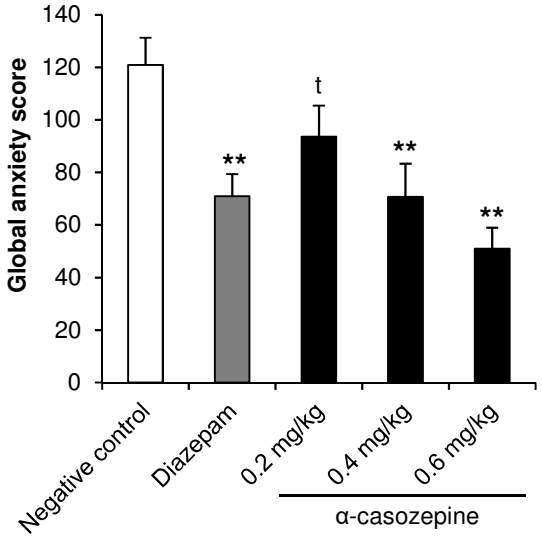


*Figure 2. Global anxiety score for rats in the CDB test after i.p. administration of α-casozepine (0.2, 0.4 and 0.6 mg/kg BW), diazepam (1 mg/kg BW) or the vehicle (negative control). Data are mean ± SEM. Unpaired t-test (2-tail.): t p<0.1; **p<0.01 (vs. negative control)*

Conclusion

α-casozepine intraperitoneally administered at 0.4 and 0.6 mg/kg body weight, induced a significant anxiolytic-like effect comparable to diazepam at 1.0 mg/kg body weight, in the CDB model in male Wistar rats.

Duration of the anxiolytic-like effect of α-casozepine

Test aim

Using the CDB test, the study aimed at evaluating the duration of the anxiolytic-like effect of i.p. administered α-casozepine.

Treatment

α-casozepine was tested at a dose of 0.6 mg/kg body weight (BW) dissolved in 0.9% NaCl solution. It was intraperitoneally administered 4 or 6 hours before the test started. Control animals received the same volume of NaCl solution (negative control).

Results

At a dose of 0.6 mg/kg body weight administered 4 hours prior to the test, α-casozepine significantly decreased the global anxiety score in comparison to the negative control (Fig. 3).

Administered 6 hours before the test, a tendency in the decrease of the global anxiety score was observed when compared to the negative control.

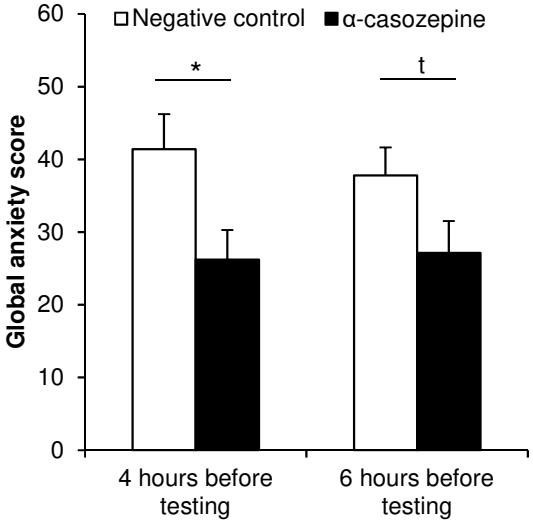


*Figure 3. Global anxiety score for rats in the CDB test after i.p. administration of α-casozepine (0.6 mg/kg BW) or the vehicle (negative control) 4 and 6 hours prior to the test. Data are mean ± SEM. Unpaired t-test (2-tail.): t p=0.1; *p<0.05 (vs. negative control)*

Conclusion

α-casozepine, intraperitoneally administered at a dose of 0.6 mg/kg body weight 4 hours before the test, showed a significant anxiolytic-like effect in the conditioned defensive burying model in male Wistar rats.

Anxiolytic-like effect of orally administered Lactium®

Test aim

The study aimed at evaluating the anxiolytic-like effect of Lactium® orally administered (p.o. administration), in the CDB test.

Treatment

Lactium® was studied at a dose of 15 mg/kg body weight (BW). It was dissolved in 0.9% NaCl solution and orally administered 60 minutes before the test started. Animals in the negative control group received the same volume of NaCl solution (vehicle). Diazepam (3.0 mg/kg body weight) was used as positive control under the same conditions.

Results

Both Lactium® and diazepam significantly decreased the global anxiety score in comparison with the negative control (Fig. 4).

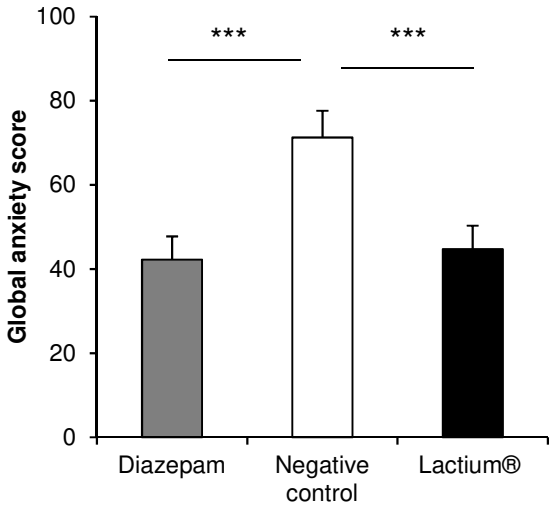


*Figure 4. Global anxiety score for rats in the CDB test 60 minutes after p.o. administration of Diazepam (3.0 mg/kg BW), vehicle (negative control) or Lactium® (15 mg/kg BW). Data are mean ± SEM. Unpaired t-test (2-tail.): ***p<0.001 (vs. negative control)*

Conclusion

Lactium®, orally administered at a dose of 15 mg/kg body weight, showed a significant anxiolytic-like effect, equivalent to that of diazepam at 3 mg/kg body weight, in the CDB test.

Anxiolytic-like effect of orally administered Lactium® evaluated in the Elevated Plus Maze model

Test aim

The study aimed at evaluating the anxiolytic-like effect of Lactium® orally administered (p.o. administration), using the elevated plus maze (EPM) test in male Wistar rats.

Treatment

Lactium® was studied at a dose of 15 mg/kg body weight and diazepam (positive control) at a dose of 3.0 mg/kg body weight. They were dissolved in 0.3% methyl cellulose and orally administered 60 minutes before test started. Control animals received the same volume of methyl cellulose (vehicle).

Results

In both Lactium® and diazepam treated rats, significant increases were observed in the percentage of open arm entries (Fig. 5A) and in the percentage of the time spent in the open part of the maze (Fig. 5B) compared to the negative control.

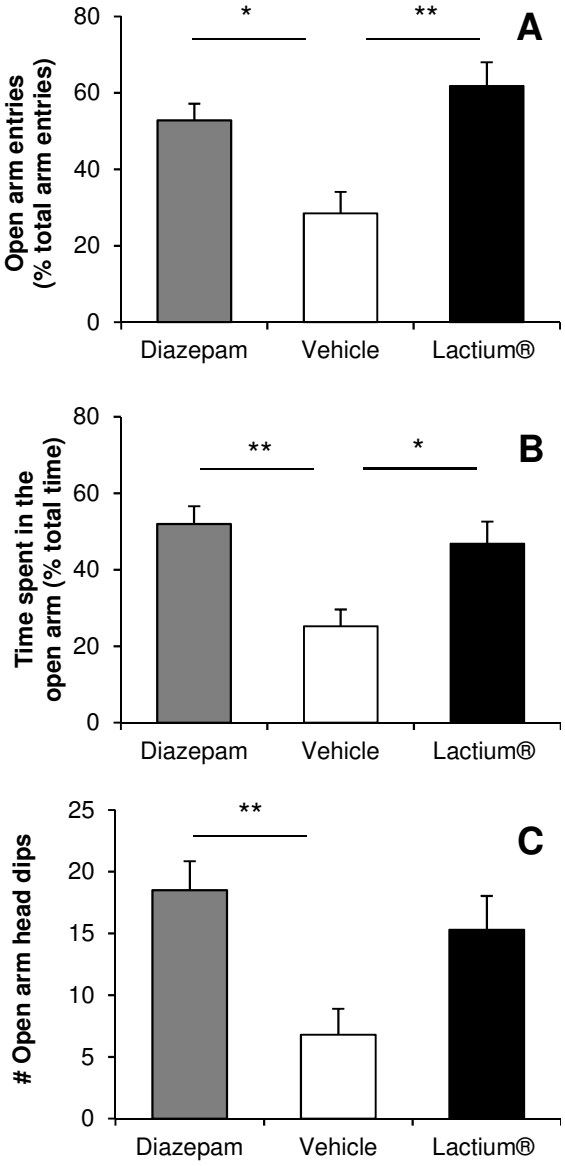


*Figure 5. Percentage of open arm entries (A), percentage of time spent in the open arm (B) and number of open arm head dips of rats in the EPM after p.o. administration of diazepam (3.0 mg/kg BW), vehicle (negative control) or Lactium® (15 mg/kg BW) 1 hour before the test. Data are mean ± SEM. ANOVA with Scheffé's test for post-hoc analysis was used: *p<0.05; **p<0.01 (vs. negative control)*

However, in the total number of head dips only diazepam induced a significant increase compared to controls whereas administration of Lactium® did not (Fig. 5C).

Head dips, which are related to vertical exploration of the maze in the open arms, induce a high risk of falling out of the maze and represent a risk taking behaviour. This suggests that Lactium® did not induce a state of disinhibition in rats as it is the case with diazepam.

Conclusion

Lactium®, orally administered at a dose of 15 mg/kg body weight, is acting as an efficient anxiolytic compound comparable to diazepam in the EPM test. However, diazepam seems to induce a state of disinhibition in rats, whereas Lactium® does not.

Reference

Violle N., Messaoudi M., Lefranc-Millot C., Desor D., Nejdi A., Demagny B., Schroeder H. *Ethological comparison of the effects of a bovine αs1-casein tryptic hydrolysate and diazepam on the behaviour of rats in two models of anxiety.* Pharmacol Biochem Behav 2006, 84 (3), 517-523.

Lactium® mechanism of action evaluated in preclinical study

The goal of the present study was to examine the Lactium® mechanism of action in the conditioned defensive burying (CDB) test using male Wistar rats. The test is described in detail in appendice 1.

The rats used in the present study were treated according to the rules provided by the ASAB (Association for the study of Animal Behaviour) Ethical committee (Guidelines for the use of animals in research, 2006) and the Canadian Council on Animal Care (2003).

The following studies were carried out at:

ETAP-LAB
13 rue du Bois de la Champelle
54500 Vandoeuvre-lès-Nancy (France)

Anxiolytic-like effect of orally administered Lactium® with a benzodiazepine antagonist.

Test aim

Using the CDB test, the study aimed at determining the Lactium® mechanism of action. Benzodiazepine site of $GABA_A$ receptor is a major target for the modulation of anxiety in both animals and humans. The objective is to evaluate whether Flumazenil, a benzodiazepine antagonist, inhibited the anxiolytic effect of Lactium® in CDB test of anxiety in rat.

Treatment

Six groups of rats were studied (n=14). The first treatment was administered 80 minutes before testing.
Each rats received either Flumazenil (FLU; 10mg/kg of body weight (BW), dissolved in 0.9% NaCl solution) or placebo (0.9% NaCl solution; negative control) by intraperitoneal injection (I.P).
Then 60 minutes before testing, a second treatment of Lactium® (15 mg/kg of BW, dissolved in 0.9% of NaCl solution), Diazepam (benzodiazepine agonist; 3 mg/kg of BW) or vehicle (0.9% NaCl solution; negative control) was given to each of the rats by oral administration (P.O.).

Results

The administration of Lactium® at 15mg/kg P.O. 60 minutes before the CDB test induced a significant decrease of Global Anxiety Score of the rats compared to placebo, which confirms the anxiolytic effect of Lactium®.

The administration of FLU at 10mg/kg I.P. 20 min before the administration of Diazepam or Lactium® blocked their respective anxiolytic effects, but had no anxiogenic effect when injected alone.
This indicates that Lactium® exerts its anxiolytic activity via the benzodiazepine site of $GABA_A$ receptor, similarly to benzodiazepines like Diazepam.

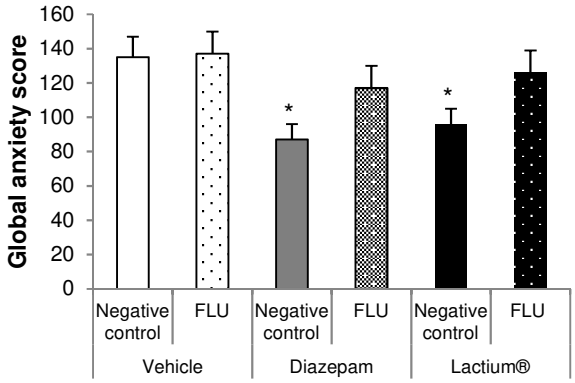


*Figure 6. Global anxiety score for rats in the CDB test: 80min after i.p. administration of FLU (10mg/kg BW) or placebo (negative control) and 60 minutes after p.o. administration of vehicle (negative control), Diazepam (3.0 mg/kg BW), or Lactium® (15 mg/kg BW). Data are mean ± SEM: test of Kruskal-Wallis following the test of Mann-Whitney *p<0.05 (vs. vehicle)*

Conclusion

The anxiolytic effect of Lactium® is mainly linked to the modulation of GABA system via benzodiazepine site of $GABA_A$ receptor. The way that Lactium® components reach the brain remains to be evaluated.

Reference

Boulier A., Violle N. *Evaluation of the mechanism of action of LACTIUM®, a milk hydrolysate enriched in alpha-casozepine with anxiolytic properties*. NUTRITION 2018

Clinical trials evaluating efficacy of Lactium® on stress and anxiety

Effects of Lactium® on acute mental and physical stress in healthy humans

Study aim

The aim of this clinical investigation was to evaluate the protective properties against stress of 3 x 400 mg of Lactium® within 24 hours prior to a psychological test (Stroop test of colour conflict) followed by a physical test (hand immersion in cold water) (Appendix 3) by measuring blood pressure and heart rate.

Study facility

Clinical Investigation Center of Necker-Enfants Malades Hospital
149 rue de Sèvres
75015 Paris (France)

Study protocol

In a parallel, placebo-controlled study design, 42 male participants were randomly assigned to one of the two study groups. On the day prior to the stress test, they ingested two 200mg capsules of either Lactium® or placebo (skimmed milk powder) in the morning and two in the evening. On the morning of the study day, 90 to 120 minutes after having taken two more 200mg capsules, the test started and volunteers were asked to remain seated for the entire time of the experiment.

During the experiment systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (HR) were measured. Recordings started with a 5 minute rest period followed by a 5 minute stress period induced by the Stroop test (Appendix 3). Subsequently, a 5 minute recovery period was recorded. After an additional rest of 30 minutes, the second test started with a 5 minute recording of a rest period, then a 5 minute stress period induced by the cold pressure test and a 5 minute recovery period. Blood samples for quantification of ACTH (adrenocorticotropic hormone) and cortisol levels were collected before the tests and after the rest phase after the second test.

Study results

Stroop test (mental stress):

During the stress period, systolic and diastolic blood pressure increased significantly less in the Lactium® compared to the placebo group (Fig. 6). Heart rate was significantly increased in both treatment groups but the increase was not different between the groups.

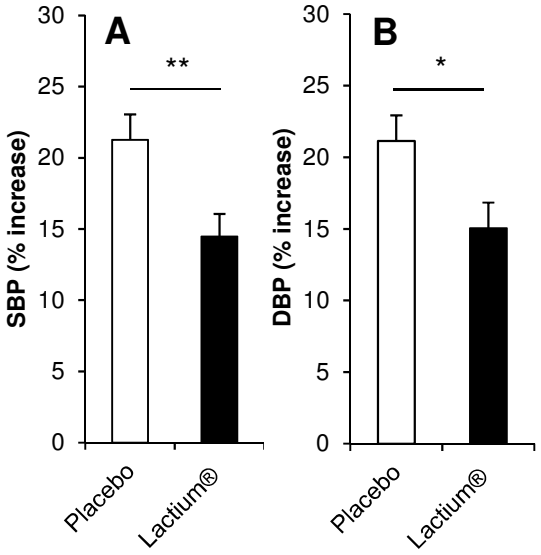


*Figure 7. Increase of systolic (A) and diastolic (B) blood pressure during the Stroop test. Data are mean ± SEM. Unpaired t-test (2 tail.): ** p<0.01 *p<0.05 (Lactium® vs. placebo)*

Cold pressure test (physical stress):

When analysed independently from the Stroop test, systolic and diastolic blood pressure of volunteers increased significantly during the stress period but this increase did not differ between the two treatment groups. Heart rate remained stable in response to the stress in the Lactium® group but increased significantly in the placebo group.

When compared to the rest value before the Stroop test, systolic and diastolic blood pressure significantly increased for both the Lactium® and the placebo group (Fig. 7) with a significantly higher increase of the systolic blood pressure in the placebo group.

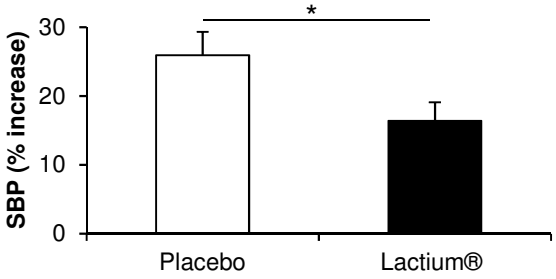


*Figure 8. Increase of systolic blood pressure during the Cold pressure test. Data are mean ± SEM. Unpaired t-test (2 tail.): *p<0.05 (Lactium® vs. placebo).*

Plasma cortisol levels were significantly reduced after the stress tests in the Lactium® treated group but not in the placebo group (Fig. 8). ATCH and cortisol concentrations were not affected by the treatment.

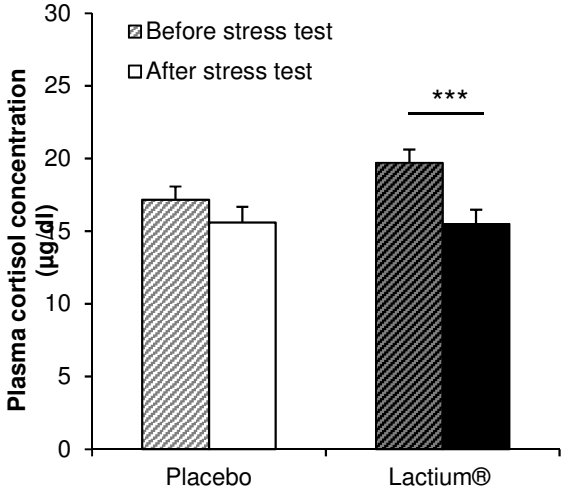


*Figure 9. Change in plasma cortisol concentration before and after the stress tests. Data are mean ± SEM. Paired t-test: *** p<0.005 (before vs. after stress test)*

Conclusion

A high acute dose of Lactium® (3 x 400 mg within 24 hours) significantly reduced the increase in blood pressure in response to a mental stress, but not to a physical stress. Therefore, it can be assumed that Lactium® shows a protection from situations of psychological stress.

This study was presented at the *4th World Congress on Stress* in Edinburgh, UK.

References

Messaoudi M., Bresson J.-L., Desor D., Lefranc C., Boudier J.-F. & Paquin P. *Anxiolytic-like effects of the milk protein hydrolysate Lactium® in healthy human volunteers.* Stress 2002, 5 (suppl.), 124.

Messaoudi M., Lefranc-Millot C., Desor D., Demagny B., Bourdon L. *Effect of a tryptic hydrolysate from bovine milk αs1-casein on hemodynamic responses in healthy human volunteers facing successive mental and physical stress situations.* Eur J Nutr 2004, 44 (2):128-32.

Effects of repeated ingestion of Lactium® on blood pressure reactivity to a mental stress

Study aim

The aim of the study was to investigate the effects of a daily ingestion of 150 mg Lactium® for 30 days by healthy participants on the basal psycho-physiological state and psycho-physiological reactions in response to a mental stress.

Study facility

Centre de recherches du service de santé des armées (CRSSA)
24 Avenue Maquis du Grésivaudan
38700 La Tronche (France)

Study protocol

Psychophysiological effects of a single 150 mg daily dose of Lactium® taken in the evening for 30 days were studied in a double-blind parallel trial. 52 healthy volunteers, 25 men and 27 women, were divided into two gender-stratified groups, Lactium® and placebo (Fig. 9). Participants were subjected to 4 identical tests prior to the first supplementation (D0), after 10 days and 30 days of supplementation (D11 and D31, respectively) and after 12 days of wash-out (D43).

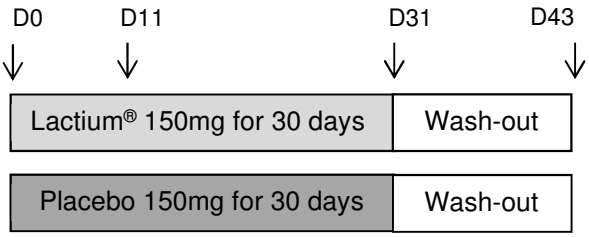


Figure 10. Study design. ↓ Test days.

On every test day, prior to the start of the experimental procedure, the baseline psycho-physiological condition was assessed.

- *Physiological assessments*: systolic and diastolic blood pressure, heart rate, night urinary cortisol
- *Psychological assessments*: Trait-STAI, Cohen PSS14 test, Vitaliano test.

Each experiment consisted of a five minute rest period followed by five minutes of exposure to mental stress and finally a five minute recovery period. Mental stress was induced by a cognitive conflict (Stroop test) (Freyschuss et al., 1990).

At the end of each of the three periods, physiological and psychological markers were assessed again.

- *Physiological assessments*: systolic and diastolic blood pressure, heart rate, salivary cortisol
- *Psychological assessments*: Strait-STAI, Thayer Activation-Deactivation

Stress reactivity was defined as the difference between the recovery value (end of the recovery period) and the stress value (first minutes of the Stroop test period). Side effects were evaluated using the Hopkins checklist after 30 days of nutriment consumption.

Study results

Entire study population:

No significant difference on cardiovascular parameters or on psychological parameters was detected at baseline. Before the beginning of the treatment (D0), during the stress period, heart rate was significant higher in the Lactium® compared to the placebo group. This significant difference disappeared during the treatment. Over the entire treatment period, stress reactivity in diastolic blood pressure (DBP) was significantly smaller in the Lactium® treated participants (p=0.049) (Fig. 10).

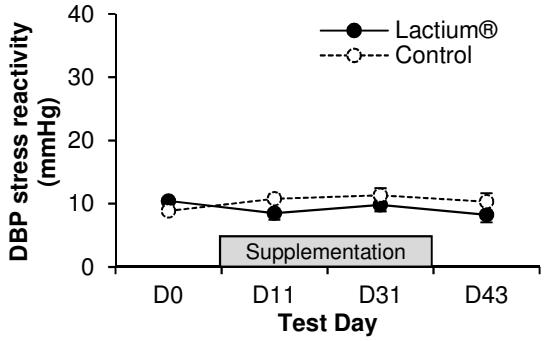


Figure 11. Stress reactivity of the diastolic blood pressure. Data is expressed as mean ± SEM.

High and low responders:

A cluster analysis of the variables systolic blood pressure stress reactivity and trait-STAI at D0 allowed grouping the study population in low and high responders. As a result, high responders (n=14) had a significantly higher systolic and diastolic blood pressure as well as heart rate during the stress period compared to the low responders (n=37). One person dropped out of the study.

Taking into account stress sensitivity (high responders), stress reactivity of systolic blood pressure (SBP) and diastolic blood pressure (DBP) was significantly lower in the Lactium® group when analysed over the entire treatment period (p=0.023 and p=0.039, respectively) (Fig. 11).

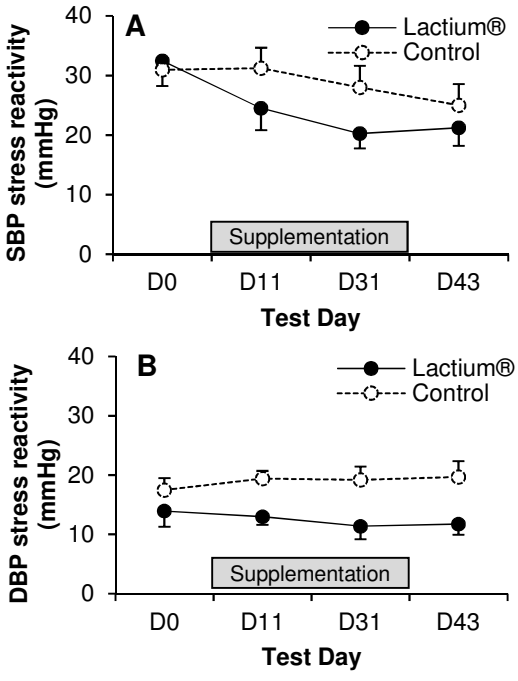


Figure 12. Stress reactivity of the systolic (A) and the diastolic (B) blood pressure in high responders. Data is expressed as mean ± SEM .

Psychological parameters were not modified in this study. No side effects were observed after a 30-day consumption of the nutriment, or 12 days after having stopped the nutriment consumption.

Conclusion

Consumption of 150 mg of Lactium® daily over a period of 30 days significantly reduced the stress reactivity of diastolic blood pressure in response to a mental stress. Focusing only on high responders by taking into account stress sensitivity, allowed to detect a reduction in stress reactivity in both systolic and diastolic blood pressure.

This study was presented at the *4th World Congress on Stress* in Edinburgh, UK.

Reference

Lanoir D., Canini F., Messaoudi M., Lefranc C., Demagny B., Martin S. & Bourdon L. *Long term effects of a bovine milk αs1-casein hydrolysate on healthy low and high stress responders.*Stress 2002, 5 (suppl.), 124.

Anti-stress efficacy of Lactium® on chronically stressed women

Study aim

The aim of this study was the evaluation of the effect of a daily ingestion of 150 mg Lactium® during 30 days upon stressed women, namely the reduction of their stress, more particularly in its various tenseness aspects.

Study Facility

Proclaim
Parc d'activité de la Bretèche
35760 Saint-Grégoire (France)

Study protocol

The trial was carried out in a double-blind, crossover design with 30 days of treatment followed by a 21 days washout period and a subsequent second 30 days treatment period. 63 women showing a sign of stress in at least one of the investigated functions received the daily dose of 150 mg Lactium® or the placebo in a randomised order.

Efficacy of the treatment was evaluated by a questionnaire in which the seriousness of each symptom was indicated on a 10-degrees scale (0=not at all, 9=excessively).

The questionnaire investigated the following main areas with their major symptoms potentially affected by stress:

- Physical and physiological area (possible troubles of digestive tract, respiratory system, cardiovascular system, locomotor system, other physical symptoms)
- Psychological area (possible troubles of intellectual and emotional functions)
- Social life (troubles in social relations)

The participants answered the questionnaire on the 1st, 15th and 30th day of each treatment period.

Definition of the "Major Symptom"

Responses to stress can be different between individuals, but constant for a given one. This is called "stereotypy of individual response" (Rosenzweig, Leiman & Breedlove, 1998).

Someone who suffers from stress mainly hopes for a decrease in his most unpleasant symptoms. Consequently, the effect of Lactium® was searched relatively to the major symptom of each area. In case of tied values, the maximum variation was selected.

Study results

Lactium® treatment at the dose of 150 mg/day was particularly efficient on the volunteers who showed high intensities (> 4) for their major symptoms. This was the case both after 15 and 30 days of treatment. There was also an effect of the placebo treatment.

Some effects of Lactium® could already be detected on the 15th day of treatment. Significantly higher improvements in the Lactium® compared to the placebo group were observed on digestive system, cardiovascular system and on some physical troubles for the participants who showed high initial intensities for their major symptoms (>4 at D0).

After 30 days of treatment, Lactium® induced significant improvements in eight areas in volunteers who showed high intensities for their major symptoms (Fig. 12). When comparing the Lactium® to the placebo group, a significantly greater evolution of the symptoms was demonstrated in 5 areas: digestive troubles, cardiovascular troubles, intellectual troubles, emotional problems and social troubles.

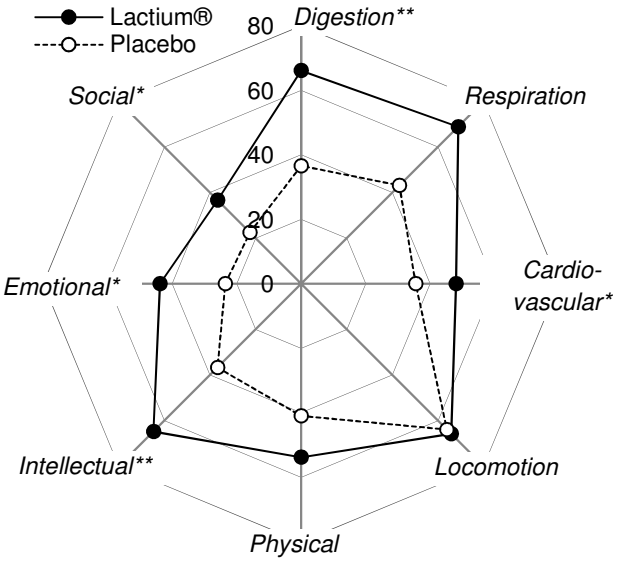


*Figure 13. Improvement of the major symptoms from day 0 to day 30. Data are mean. *p<0.05; **p<0.01 (Lactium® vs. placebo)*

These results suggest regulating properties of Lactium® in the field of stress-linked troubles (tension):

- When the tension is related to various physiological systems (here: cardio-vascular and digestive systems).
- When the tension is perceived as an internal state that negatively interacts with the affective (emotional troubles), the intellectual and the relational functions (social behaviour) of the participants.

Conclusions

Lactium® can reduce stress related symptoms when the tension is resulting in physiological troubles (cardiovascular- and digestive system) as well as in affective (emotional troubles), intellectual and the relational functions (social behaviour).

Reference

Kim J.H., Desor D., Kim Y.T., Yoon W.J., Kim K.S., Jun J.S., Pyun K.H., Shim I. *Efficacy of αs1-casein hydrolysate on stress-related symptoms in women.* Eur J Clin Nutr. 2007; 61 (4):536-41.

Burnout: Evaluation of the efficacy and tolerability of a dietary supplement containing Lactium® for professional fatigue syndrome (burnout)

Study aim

The aim was to study the effect of a dietary supplement containing Lactium® on the symptomatology of burnout.

Study facility

Department of Clinical Pharmacology
University of Bordeaux Ségalen
33076 Bordeaux (France)

Study protocol

A 12-week, double-blind, randomized, placebo-controlled trial was conducted in 85 male and female workers engaged in professional contact with patients, students or clients. All were affected by burnout syndrome based on a score of ≥4 on the Burnout Measure Scale (BMS-10).

Participants consumed daily either the dietary supplement with 150 mg/day of Lactium® or the placebo. Next to Lactium® the dietary supplement also contained taurine (100 mg/day), an extract of *Eleutherococcus senticosus* (100 mg/day) and a melon extract (10 mg/day). It is licensed under the name TARGET 1®.

In addition to the supplements, participants had three visits with the primary investigator, a medical doctor. At each study visit, participants underwent interviews that discussed their family, social and professional relationships.

The primary outcome measure was the change in the BMS-10 score; secondary outcome measures included the change in the Maslach's Burnout Inventory scale-Human Service Survey (MBI-HSS) score and the Beck Depression Inventory. In addition, a questionnaire evaluating the patients quality of life was included. Evaluation instruments were administered at 3 visits: at inclusion, after 42 days of treatment and after 84 days of treatment.

Results

After 12 weeks of supplementation, the placebo group showed significant improvements in scores for BMS-10, MBI-HSS fatigue and the Beck Depression Inventory, but MBI-HSS depersonalization and task management were not improved; the group receiving the supplement containing Lactium® showed significant improvements in all five scores. Life quality scores were also highly significantly improved (Fig. 13). The group receiving the supplement containing Lactium® consistently showed significantly greater improvements in scores than the placebo group.

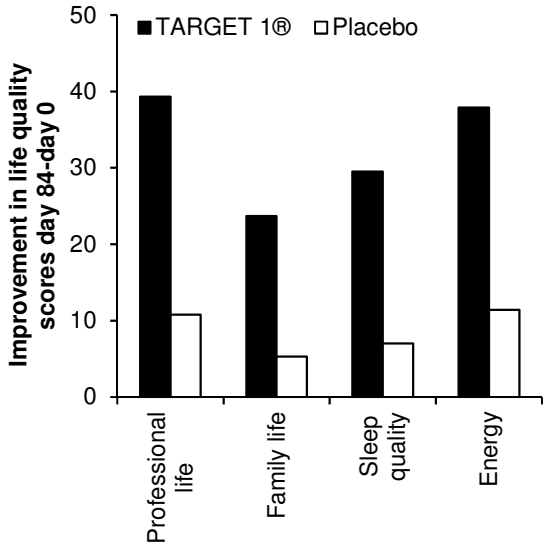


Figure 14. Improvement in visual analog scale scores for life quality between inclusion (day 0) and after 84 days of treatment. Data is expressed as mean.

Conclusion

In combination with verbal expression, TARGET 1® containing Lactium® significantly reduces the symptoms of burnout after 12 weeks' use.

According to the study authors, these data were presented orally at the P2T Congress.

(Pharmacology, Physiology, Therapeutics), Poitiers, France, 2014. Abstract number: CO-109.

Reference

Jacquet A., Grolleau A., Jove J., Lassalle R., Moore N. *Burnout: Evaluation of the efficacy and tolerability of Target 1® for professional fatigue syndrome (burnout).* J Int Med Res. 2015 Feb; 43(1):54-66.

Relief of stress induced sleep troubles studied in rodent models

Lactium® improves sleep in rats subjected to chronic mild stress

Study aim

This study aimed at investigating if Lactium® can improve sleep troubles induced by a mild chronic stress.

Study facility

UMR INRA 914 Physiologie de la Nutrition et du Comportement Alimentaire
Institut National Agronomique Paris-Grignon
16 rue Claude Bernard
75231 Paris Cedex 05 (France)

Study protocol

Male Wistar rats were subjected to chronic stress during 8 days in the form of environmental disturbances (sonorous disturbances, light–dark cycle disturbances and a different tilt to the cage every 12 h). At the same time they received either an oral administration of 15mg/kg body weight Lactium® or total milk protein (negative control).

Sleep patterns were monitored at baseline and at day 1, 2, 4 and 8 of the treatment period by Electroencephalography (EEG).

Results

Total sleep duration was significantly decreased in the control group, mostly due to a significant shortening in the duration of slow wave sleep (deep sleep) during day 2 of the mild stress procedure (Fig. 14). Lactium® treated rats were preserved from this decrease. Moreover, despite the pursuit of the stress procedure, sleep gradually recovered in the control group and was no longer significantly affected after 4 days.

Conclusion

Lactium® can assist in the prevention of stress-related disturbance in slow wave sleep and total sleep duration in mildly stressed rats.

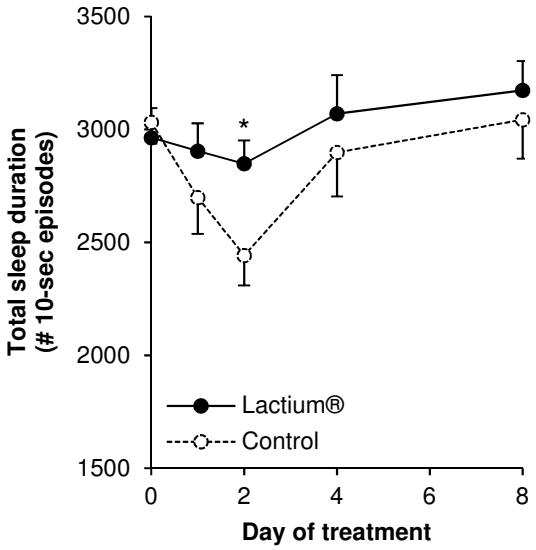


*Figure 15. Evolution of total sleep duration during the eight days treatment period. Data are mean ± SEM. Unpaired t-test (2-tail.): *p<0.05 (vs. negative control)*

Reference

Guesdon B., Messaoudi M., Lefranc-Millot C., Fromentin G., Tomé D., Even P.C. *A tryptic hydrolysate from bovine milk αs1-casein improves sleep in rats subjected to chronic mild stress.* Peptides 2005, 27 (6):1476-82.

Lactium® enhances medically-induced sleep in mice and brain patterns of sleep in rats

Study aim

The study aimed to investigate the potential sedative and sleep-promoting effects of Lactium® in a rodent model.

Study facility

Uimyung Research Institute for Neuroscience
Department of Pharmacy
Sahmyook University
815 Hwarang-ro
Nowon-gu
Seoul 139-742 (Republic of Korea)

Study protocol

Male mice received orally different concentrations of Lactium® (75, 150, 300, or 500 mg/kg body weight). The vehicle was used as negative control. Diazepam administered intraperitoneally at a concentration of 1 mg/kg

body weight acted as positive control. One hour after administration, sedative and sleep promoting effects were assessed after a single administration and after repeated administration during 5 days. Sedative effects were measured using the open field (locomotor activity) and the rota-rod test (test of motor coordination). Sleep promoting effects were investigated by recording the time to fall asleep and the sleep duration after injection of pentobarbital, a sleep-inducing drug.

Brain waves were monitored using EEG in Sprague-Dawley rats which had received 150 or 300 mg/kg body weight of Lactium®.

Results

In contrast to diazepam which reduced locomotor activity and performance of mice on the rota-rod, Lactium® did not show any sedative effects compared to the negative control.

Lactium® did not reduce the time for the onset of sleep in response to pentobarbital injection. In contrast, a single dose of 150 mg/kg body weight of Lactium® as well as the same dose administered daily for 5 days significantly increased sleep duration in mice compared to the negative control.

In rats, Lactium® at a dose of 150 mg/kg body weight increased slow wave delta EEG activity significantly compared to the negative control. This is a pattern which indicates sleep or relaxation.

Conclusion

The results suggest that Lactium® has sleep promoting effects in rodents without being a sedative.

Reference

Dela Peña I.J., Kim H.J., de la Peña J.B., Kim M., Botanas C.J., You K.Y., Woo T., Lee Y.S., Jung J.C., Kim K.M., Cheong J.H. *A tryptic hydrolysate from bovine milk αs1-casein enhances pentobarbital-induced sleep in mice via the GABAA receptor.* Behav Brain Res. 2016 Jul 9;313:184-190.

Stress related sleep troubles investigated in clinical trials

Effect of Lactium® on sleep disturbances in Japanese general population

Study aim

This trial intended to evaluate the efficacy of Lactium® 150 mg intake on sleep quality.

Study facility

Institute of General Health Development Co. Ltd.
SHIBA Palace Clinic
SHIBA-DAIMON 105-0012 Tokyo (Japan)

Study method

A total of 44 volunteers who had reported sleeping troubles and stress at work completed this randomized, double blind, placebo-controlled, parallel trial. Participants consumed either 150 mg of Lactium® or a placebo for 4 weeks in total, succeeded by a follow-up period of one week. Sleep was evaluated using the Epworth Sleepiness Scale (ESS) (Johns, 1991) and the Pittsburgh Sleep Quality Index (PSQI) (Buysse *et al.*, 1989). Questionnaires were at baseline (before intake of treatment, D0), after two weeks (D14), after four weeks (D28) and after the follow-up week (D35).

Study results

Epworth Sleepiness Scale (ESS)

Lactium® significantly reduced the risk of dozing in observational situations after 4 weeks of treatment and this effect was still observed until 1 week after the intake had stopped.

Pittsburgh Sleep Quality Index (PSQI)

Sleep quality was significantly improved after 2 and 4 weeks of Lactium® as well as placebo ingestion (Fig. 15). This effect remained even 1 week after the intake had stopped.

When looking at the subset of female participants (n=32), sleep quality was significantly improved in the Lactium®, but not in the placebo group.

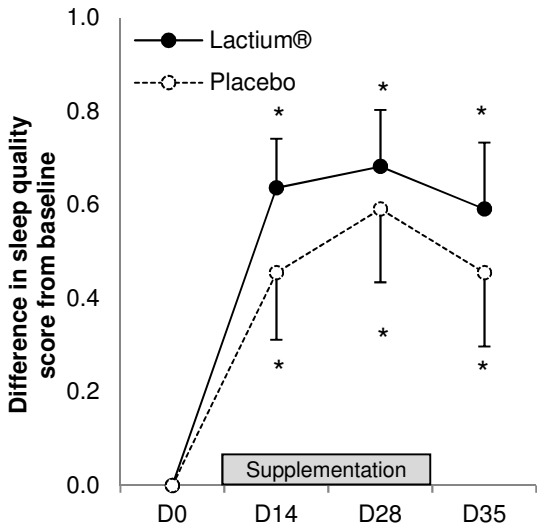


*Figure 16. Improvement in sleep quality scores of participants. Data are mean ± SEM. Wilcoxon signed-rank test: *p<0.05/3 (vs. baseline value)*

No significant difference was seen between Lactium® and placebo-treated participants for both questionnaires.

Conclusion

Lactium® at a dose of 150 mg per day significantly improves sleep quality in participants who are aware of sleep disorders. Given the anti-stress properties of Lactium®, it seems possible to relate the detected improvement of sleep aspects to a reduction of stress following its chronic administration.

References

Buysse D.J., Reynolds III C.F., Monk T.H., Berman S.R. & Kupfer D.J. *The Pittsburgh Sleep Quality Index: A New Instrument for Psychiatric Practice and Research.* Journal of Psychiatric Research 1989, 28(2), 193-213.

De Saint Hilaire Z., Messaoudi M., Desor D., Kobayashi T. *Effects of a bovine α_{s1}-casein tryptic hydrolysate (CTH) on sleep disorder in Japanese general population.* The Open Sleep Journal 2009, 2, 26-32.

Johns M.W. *A new method for measuring daytime sleepiness: the Epworth sleepiness scale.* Sleep 1991; 14 (6), 540 – 545.

Effect of Lactium® on sleep disturbances in poor Korean sleepers

Study aim

This trial intended to evaluate the efficacy of Lactium® 300mg intake on sleep quality.

Study facility

Sleep Center, Ewha Womans University Mokdong Hospital, Seoul, Korea

Study method

A total of 48 volunteers who had reported sleeping troubles with the Pittsburgh Sleep Quality Index (PSQI>5) completed this randomized, double blind, placebo-controlled, cross-over trial. Participants consumed either 300 mg of Lactium® or a placebo during the initial 4 weeks of phase I; after a 4-week washout period, the counterpart capsule was administrated during the 4 weeks of phase II in the exact same manner.

Sleep was evaluated using the PSQI, the Insomnia Severity Index (ISI) in the monitoring phase and the screening phase. Subjective excessive daytime sleepiness (EDS) was measured using the Epworth Sleepiness Scale (ESS). The fatigue was measured using the Fatigue Severity Scale (FSS). Depression and anxiety symptoms were measured with the Beck Depression Inventory (BDI) and Beck Anxiety Inventory (BAI).

Participants were asked to record 8 weeks of a sleep diary regarding the times when they went to sleep and woke up in order to determine time in bed (TIB), time taken to fall asleep (SL), subjective total sleep time (TST), number and time of each waking after sleep onset (WASO). Daily sleep diary data were averaged for one week, and sleep efficiency (SE) was calculated as the percentage of TST compared to total TIB.

For the objective parameter, actigraphy was used on the nondominant hand during the 8 weeks of recording for both phase I and phase II. The variables measured were TIB, SL, TST, SE, and WASO. The standard for normal SE was set at superior at 85%.

Full-night polysomnography PSG was also performed before and after each phase on 24 subjects who agreed to the one-night stay at the sleep laboratory to take the sleep test in the hospital.

Study results

Sleep & Mood questionnaire scales

Lactium® and placebo groups showed a gradual decrease in PSQI score after 4 weeks compared with the baseline score. The sleep discomfort index (measured by ISI) was similar, with a significant effect of time ($p < 0.001$). No difference was found in ESS, FSS, BDI and BAI questionnaires.

Subjective and objective sleep profile monitoring: sleep diary and actigraphy

TIB was decreased in both placebo and Lactium® groups without statistical significance. TST increased and SL decreased in the Lactium® group, indicating an improvement of sleep quantity. Significant differences were observed in the sleep diary assessment, but not in the actigraphy assessment (TST, $p < 0.001$, $q < 0.001$ vs. $p = 0.270$; SL, $p < 0.001$, $q < 0.001$ vs. $p = 0.063$).

The ratio of participants with a tendency toward improved sleep quantity was also higher in the Lactium® group compared to the placebo group for both sleep diary and actigraphy measures.

SE was significantly improved in the Lactium® group compared to the placebo group in both measures (Figure 17) after 4 weeks of supplementation ($p < 0.001$, $q < 0.001$ for sleep diary and $p = 0.007$, $q = 0.016$ for actigraphy). The ratio of participants with increased SE was also higher in the Lactium® group compared to placebo (85% vs. 24%, $p < 0.001$, $q < 0.001$ for sleep diary; 85% vs. 63%, $p = 0.031$, $q = 0.078$ for actigraphy).

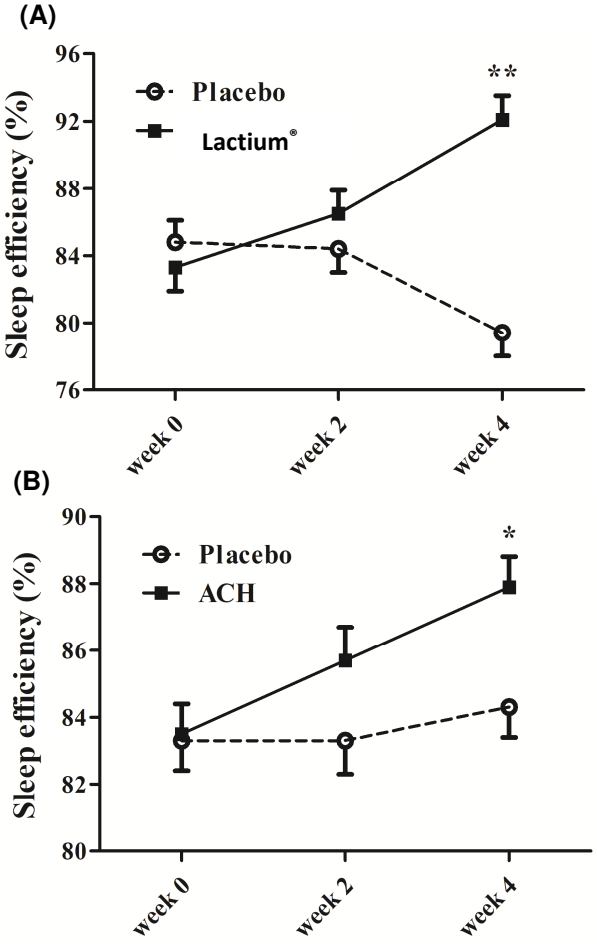


*Figure 17. Comparison of effects of Lactium® administration on sleep efficiency. (A) Sleep diary. (B) Actigraphy *q-value < 0.05 and **q-value < 0.01, pFDR was calculated for group × time difference by linear mixed-effects model to account for multiple testing.*

Sleep disturbance measures determined by WASO significantly decreased. The number of awakenings showed a declining tendency in the Lactium® group; this also indicated sleep quality improvement (WASO, $p < 0.001$, $q < 0.001$ for sleep diary vs. $p = 0.053$ for actigraphy; number of awakenings, $p = 0.077$ by sleep diary vs. $p = 0.240$ by actigraphy). The ratio of participants with disturbed sleep was lower in the Lactium® group than in the placebo group for both measures.

PSG measures

No significant difference was observed between Lactium® and placebo groups. This is because the participants only spent one night at the hospital so they couldn't get used to it.

Conclusion

Lactium® at a dose of 300mg per day for 4 weeks significantly improves sleep quantity and quality among adults with low sleep quality. These results suggest that Lactium® can be safely used to help individuals with sleep disturbances.

References

Kim J.H., Kim J., Lee S., Kim B., Kwon E., Lee J.E., Chun M.Y., Lee C.Y., Boulier A., Oh S., Lee H.W., *A Double-Blind, Randomized, Placebo-Controlled Crossover Clinical Study of the Effects of Alpha-S1 Casein Hydrolysate on Sleep Disturbance*. Nutrients. 2019; 11, 1466

Improvement of insomnia symptoms with Lactium® enriched night milk

Study aim

The aim of this study was to determine whether Lactium® enriched night milk, obtained from milking cows at night and containing higher levels of melatonin, can improve symptoms of insomnia.

Study facility

WellSleep
Department of Medicine
University of Otago Wellington
Wellington (New Zealand)

Protocol

In a double blind placebo-controlled crossover trial, 19 adults with primary insomnia were randomized to 3 weeks of night milk enriched with 150mg of Lactium® per day and 3 weeks of day milk separated by a one-week washout.

Night milk was obtained by selectively collecting milk produced by cows during the night. In this way it benefits from a naturally higher content of a sleep inducing hormone, melatonin, compared to standard milk (Milagres *et al*., 2014). The Lactium® enriched night milk is commercialized under the name iNdream3 ™ by Synlait Milk Ltd.

The study assessed both objective and subjective measures of sleep obtained by in-home polysomnography, actigraphy, sleep diary and sleep questionnaire.

Results

Sleep efficiency

As measured by actigraphy, daily ingestion of iNdream3 ™ resulted in significantly improved sleep efficiency compared to both baseline and day milk (Fig. 16).

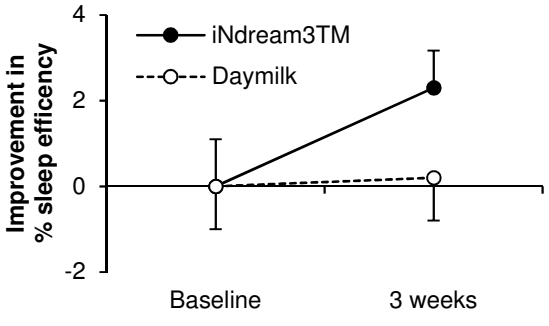


Figure 18. Improvement in % sleep efficiency after 3 weeks of treatment. Data are mean ± SEM.

Sleep efficiency is defined as the ratio of the total sleep time to the total time spent in bed.

When measured by subjective Pittsburg Sleep Quality Index (PSQI), an even stronger improvement in sleep efficiency was observed.

Daytime dysfunction:

Compared to both baseline and day milk, daytime dysfunction expressed in the PSQI questionnaire was significantly reduced after 3 weeks of use of iNdream3 ™ (Fig. 17).

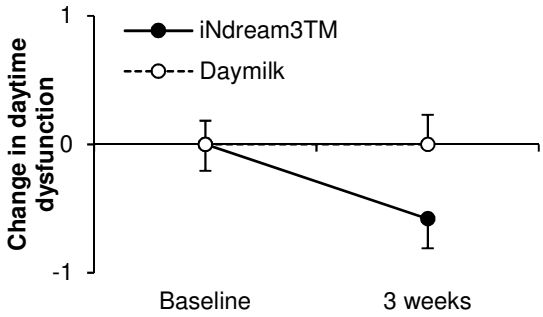


Figure 19. Change in daytime dysfunction after 3 weeks of treatment. Data are mean ± SEM.

Conclusion:

iNdream3™, a powdered milk product containing naturally increased levels of melatonin, low levels of lactose, plus Lactium®, improves both objective and subjective measures of sleep quality and daytime dysfunction.

This data was presented at the 29th Annual Meeting of the Associated Professional Sleep Societies, LLC.

References

Campell A., Neill, A. *A randomized placebo controlled trial of melatonin enriched milk – can it improve symptoms of insomnia?* SLEEP 2015, Volume 38, Abstract Supplement.

Campell A., Neill, A. *Melatonin-rich milk fortified with alpha s1 casein tryptic hydrolysate improves primary insomnia: a randomized placebo controlled trial.* Sleep Biol. Rhythms 2016, 14: 351.

Milagres M.P., Minim VP, Minim LA, Simiqueli AA, Moraes LE, Martino HS. *Night milking adds value to cow's milk.* J Sci Food Agric. 2014 Jun;94(8):1688-9

SAFETY PROFILE

Oral toxicity

Acute oral toxicity

Test aim

The aim of the study was to assess qualitatively and quantitatively the toxic effects and their delay of appearance after a single oral administration of Lactium®.

Test facility

EVIC France – Division Evic-Tox
48 rue Jean Duvert
33290 Blanquefort (France)

Test method

A stepwise procedure was used. Lactium®, dissolved in distilled water, was orally administered to female rats. As Lactium® was expected to be non-toxic, a limit test at the starting dose of 2000 mg/kg body weight was carried out with six animals (three animals per step).

After administration, animals were observed periodically during the first 24 hours, with special attention given during the first 4 hours and daily thereafter, for a total of 14 days. Signs of toxicity (mortality...) were recorded.

The acute toxic class method was used following OECD Test Guidelines 423. This test provided results allowing the test substance to be ranked and classified according to the Globally Harmonised System (GHS) for the classification of chemicals which causes acute toxicity (OECD 1998).

Results

At the dose of 2000 mg/kg body weight no signs of toxicity, no necropsy findings, no histopathological findings and no mortality were observed.

Conclusion

Lactium® was classified in the Hazard Category 5 or Unclassified with a LD50 higher than 2000 mg/kg.

Repeated dose 28-day oral toxicity

Test aim

The aim of the study was to assess qualitatively and quantitatively the toxic phenomena and the rate of onset after repeated administration of Lactium® in male and female rats.

Test facility

EVIC France – Division Evic-Tox
48 rue Jean Duvert
33290 Blanquefort (France)

Test method

Every day, for a period of 28 consecutive days, Lactium® dissolved in water was orally administered at three doses (40 mg/kg body weight, 200 mg/kg body weight, 1000 mg/ kg body weight) to a group of animals (5 males and 5 females per group), one dose being used per group. An additional control group of rats received water only.

During the administration period, the animals were closely observed each day for signs of toxicity. At the end of the treatment, biochemical and haematological examinations were performed on all animals. Animals were sacrificed to be necropsied and subjected to appropriate histopathological examinations.

The methodology followed the OECD guideline No 407 of July 27, 1995 and the Appendix IV.D part B7 of the European Directive 96/54/EEC of July 30, 1996 published in the Official Journal of the European Communities of September 30, 1996 (L248).

Results

No mortality was observed during the treatment period. Compared to the control group, in the group of rats receiving Lactium®, no significant differences were observed for body weight gain, food consumption, clinical observations, gross necropsy, organ weight and histopathology. In haematology, slight significant differences from controls were noted in treated animals (decrease in haematocrit and leucocytes, increase in prothrombin time). They were

considered to be of no toxicological importance. Compared with controls, a small, but statistically significant increase in serum ASAT and ALAT was observed in male rats receiving 200 or 1000 mg/kg/day of Lactium®. This change was not considered to be biologically significant.

Conclusion

Under the experimental conditions adopted, daily oral administration of Lactium® to male and female rats for 28 consecutive days at the doses of 40 mg/kg/day, 200 mg/kg/day and 1000 mg/kg/day induced no significant toxic effect.

In this test, the dose level of Lactium® inducing no observable toxic effects can be considered as higher than 1000 mg/kg body weight per day.

Repeated dose 90-day oral toxicity

Test aim

The purpose of the study was to establish the effects of continuous oral administration of Lactium® to rats over a period of 90 consecutive days.

Test facility

Safety Assessment Center
Korea Testing and Research Institute (KTR)
7-6, Gomak-ri, Wolgot-myeon
Gimpo-si, Kyonggi-do (Korea)

Test method

During 90 consecutive days, Lactium® was daily administered at 40 mg/kg body weight, 250 mg/kg body weight and 1000 mg/kg body weight by oral gavage to three groups of rats (10 males and 10 females per group). An additional control group received only the vehicle during the 90-days treatment period. Two recovery groups, each five males and five females, were treated with the high dose (1000 mg/kg body weight per day) or vehicle alone for 91 days and then maintained without treatment for further 14 days.

This study complied with the recommendations of the Korea Food and Drug Administration Guidelines for Safety Evaluation of Drugs (Notification No.2005-60, KFDA).

Results

There were no treatment-related mortality or clinical signs in rats treated with Lactium®. No treatment related toxicologically significant effects on body weight, food and water consumption, organ weight, urinalysis, haematology, serum biochemistry as well as necropsy and histopathological were found.

Conclusion

Oral administration of Lactium® to rats for a period of 90 days at dose levels of up to 1000 mg/kg body weight per day resulted in no toxicological effects in all animals. Therefore, the no-observable-adverse-effect-level (NOAEL) was considered to be over 1000 mg/kg body weight per day under the conditions of this study.

Mutagenicity

Mutagenicity assed in the mammalian cell (L5178Y) Mouse Lymphoma Assay

Test aim

The study aimed at evaluating the mutagenic potential of Lactium® by the observation of its effects on a mammalian cell line (L5178Y Mouse Lymphoma) in the presence and absence of a metabolic activation system.

Test facility

MDS Pharma Services
Les Oncins - BP 0118
69593 L'Arbresle Cedex (France)

Test method

The principle of this test is to detect forward mutations which functionally mutate the thymidine kinase (TK) locus present in the cells. The mutant cells are detected by their ability to grow in the presence of the pyrimidine analogue trifluorothymidine (TFT). The normal TK proficient cells are sensitive to TFT, which causes the inhibition of further cell division. In contrast the mutant cells are able to proliferate in the presence of TFT.

The test has three principal phases; exposure, expression and determination of mutants.

Exposure

Exposure to potential mutagenic agents (Lactium® up to a concentration of 5000µg/ml, positive controls MMS and CP):

- short treatment: 4 hours either with or without metabolic activation
- long treatment 24 hours, without metabolic activation

Cytotoxicity was evaluated by the relative survival (relative to the negative control) after the treatment period or by relative total growth (relative to the negative control) during the expression period.

Expression

After exposure, the cells were washed and re-suspended in a standard medium and incubated for 2 days to allow an optimal phenotypic expression of possible induced mutations.

Determination of mutants

A known number of cells were incubated for two weeks in a medium containing the selective agent (TFT) to detect mutant cells and in a medium without TFT in order to determine the clone efficiency (viability).

The mutation rate was calculated from the number of colonies in the selective medium corrected by the number of mutant colonies in the non-selective medium.

Results

In the 3 experiments (short treatment with or without metabolic activation or long treatment without metabolic activation) and over the whole range of dose levels up to 5000 µg/ml, the results did not show evidence of any cytotoxic effect of Lactium®: no precipitate was observed, no dose-related decrease in the relative survival rate or the relative total growth. In the absence of precipitate or cytotoxicity, all treated cultures were analysed for the mutant frequencies. No statistically or biologically significant increase in the frequency of mutants was noted at any of the dose levels tested. All the values of treated groups were similar to the negative control values.

Conclusion

Under the experimental conditions and according to the criteria of the test protocol, it can be concluded that Lactium® did not induce any mutagenic effect in the mammalian cell (L5178Y) Mouse Lymphoma Assay, either with or without metabolic activation when tested up to a concentration of 5000 µg/ml.

Teratogenicity

Study of the effects of the ingestion of Lactium® during pregnancy on first and second generation offspring in rats

Test aim

The aim of this study was to evaluate the influence of the oral intake of Lactium® by female pregnant Wistar rats on internal and external malformations of their first and second generation offspring.

Test facility

Centre de Recherches ETAP
13, rue du Bois de la Chapelle
54500 Vandœuvre-lès-Nancy (France)

Pathological examination

Laboratoire d'Anatomie Pathologique
Faculté de Médecine
9, Avenue de la Forêt de Haye
54505 Vandœuvre-lès-Nancy (France)

Test method

16 female rats were crossed with males; the day after fecundation, the pregnant females were randomly divided into 2 treatment groups: a group was treated with a 150mg/kg body weight daily dose of Lactium® throughout pregnancy, and the other received the same dose of skimmed milk powder for the same period (control). At birth, each litter was reduced to 8 youngs (4 males and 4 females).

First generation offspring

At the age of 3 weeks, 8 males and 8 females were randomly selected in the litters of both groups to detect any possible malformations. Studied variables were external general malformations, internal visceral malformations, skeletal malformations and histological examination.

Second generation offspring

16 Wistar females of the first generation were crossed with non-consanguineous males of the first generation, born from females of the same treatment group. At birth each litter was reduced to 8 youngs (4 males and 4 females). At the age of 18 or 19 days, 8 males and 7 females of the Lactium® group and 8 males and 8 females of the control group were randomly selected to detect any possible malformations. Studied variables were external general malformations, internal visceral malformations and histological examination.

Results

No statistical difference for any of the parameters studied was observed after macroscopic and microscopic examination between animals from the Lactium® and the control group.

Conclusion

The daily oral intake of 150 mg/kg of Lactium® by female pregnant Wistar rats during their entire pregnancy induces no internal or external malformation in their first and second generation offspring.

Behavioural toxicity

Effects of the ingestion of Lactium® throughout pregnancy on maternal behaviour and development of the offspring in rats

Test aim

The aim of the study was to evaluate the effects of Lactium®, ingested daily throughout pregnancy, on the maternal behaviour and the physical, neuromotor and behavioural development of offspring between birth and adulthood in Wistar rats.

Test facility

Centre de Recherche ETAP
13, rue du Bois de la Champelle
54505 Vandœuvre-Lès-Nancy (France)

Test method

Sixty female rats from the Wistar strain were crossed with males; the fertilised females were randomly divided into five treatment groups: a control group treated with an oral dose of 150 mg/kg body weight of powdered milk throughout pregnancy and four groups treated with an oral dose of 150 mg/kg body weight of Lactium® either every day during the first week, the second week or the third week of pregnancy, or every day throughout the three weeks of pregnancy. At birth, each litter was reduced to 8 youngs (4 males and 4 females).

The treatment effects were evaluated on the dams regarding quality of maternal behaviour (litter size, sex-ratio and test of nest construction). On the offspring, the following variables were examined between birth and adulthood: physical development (weight evolution, incisor cutting and eye opening), neuromotor development (eversion test, grip reflex, righting reflex, hanging and locomotor coordination) and behavioural development (locomotor, exploratory activity and emotional state in open-field, spatial training in the Morris water maze and operative conditioning in the aversive light stimulus avoidance test).

Results

<u>Effects on the treated dams</u>

No differences between the Lactium® treated and the control group were observed on the pregnancy duration, number of offspring, sex-ratio at birth as well as maternal and care-to-young behaviour.

<u>Effects on the offspring</u>

Offspring born from treated dams show a weight evolution, an age of incisor cutting and an age of eye opening comparable to those born from control females.

No significant difference has been shown, either for female adult rats or young rats, whatever the parameters compared between the different groups (Lactium® or placebo).

Conclusion

A daily oral intake of Lactium® by female rats at a dose of 150 mg/kg body weight during pregnancy does not alter pregnancy duration, maternal behaviour or care of offspring. It also does not induce troubles in the physical, neuromotor, behavioural and cognitive development in the offspring.

Neuro-physiological safety

The potential behavioural effects of α-casozepine were assessed on four characteristic major side effects of benzodiazepines: **addiction**, **memory impairment, tolerance** and **disinhibition**.

Test facility

Centre de Recherche ETAP
40, rue Lionnois
54000 Nancy (France)

Potential addictive effect of α-casozepine

Test aim

The study aimed to evaluate the possible addictive effect of α-casozepine, in the conditioned place preference model (Appendix 4) in male Wistar rats.

Treatment

α-casozepine was studied at a dose of 1 mg/kg dissolved in 0.9% NaCl solution. It was administered i.p. 30 minutes before every other conditioning session. Diazepam at a dose of 3 mg/kg was used as positive control and the vehicle (NaCl) as negative control.

Results

Rats in the negative control group and those treated with α-casozepine did not modify the time spent in the non-preferred compartment before and after conditioning (Fig. 18). Diazepam-treated rats significantly increased time spent in the non-preferred compartment after conditioning.

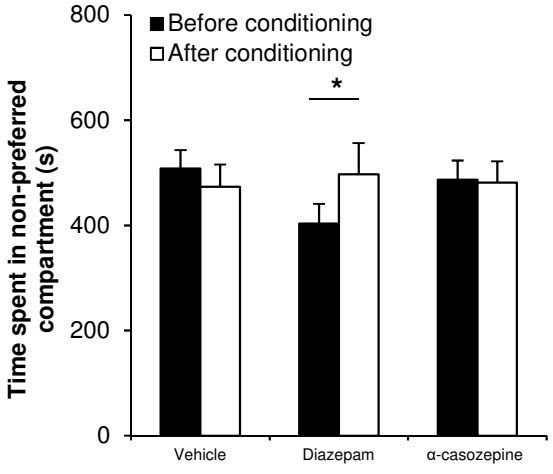


*Figure 19. Time spent in the non-preferred compartment. Data are mean ± SEM. Paired t-test (2-tail.) : * p<0.05 (before vs. after conditioning)*

Conclusion

Unlike diazepam, α-casozepine (1 mg/kg, i.p.) did not induce conditioned place preference in male Wistar rats. It can therefore be concluded that it does not have any addictive effect.

Social memory effect of α-casozepine

Test aim

The study aimed to evaluate the effect of α-casozepine on social memory in male Wistar rats.

Treatment

In the social memory test (Appendix 5) α-casozepine was studied at a dose of 0.4 and 0.8 mg/kg dissolved in 0.9% NaCl solution. It was administered i.p. to the adult rat immediately after the first exposure to a juvenile. Diazepam (2 mg/kg, i.p.) was used as positive control and the vehicle (NaCl) as negative control.

Results

Time duration of rat investigations on the first minute exposure

During the first minute, the duration of investigations of vehicle and α-casozepine-treated rats significantly decreased on the second exposure compared with the first one (Fig. 19). Rats treated with diazepam spent as much time investigating the juvenile during the first as during the second exposure.

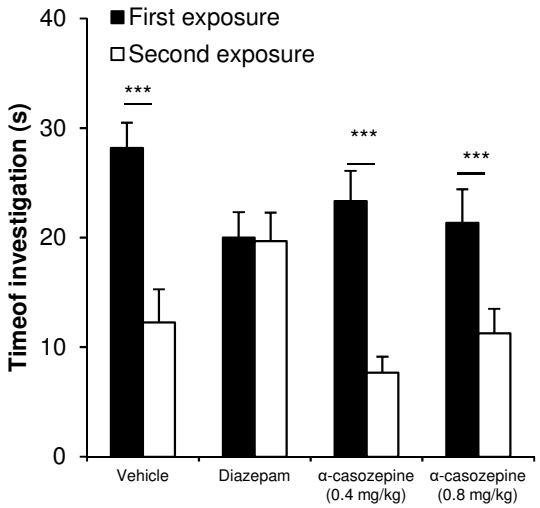


*Figure 20. Investigation time during the first minute exposure. Data are mean ± SEM. Paired t-test : *** p<0.005 (first vs. second exposure)*

Duration of rat investigations on the 5-minute exposure

The time duration of investigations of vehicle and α-casozepine-treated rats significantly decreased on the second exposure compared with the first one (Fig. 20). Rats treated with diazepam significantly increased time investigating the juvenile on the second exposure compared to the first exposure.

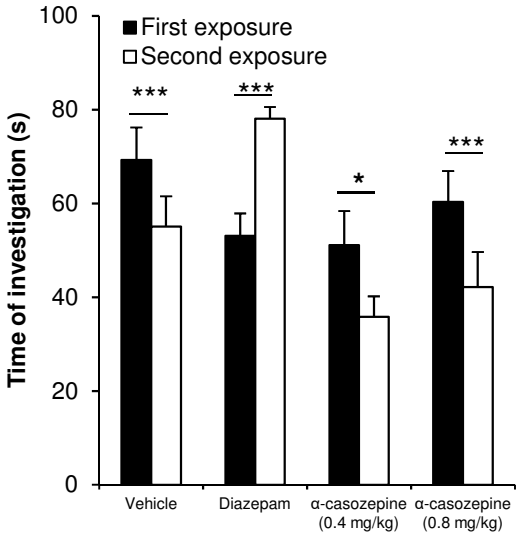


*Figure 21. Investigation time during the five minute exposure. Data are mean ± SEM. Paired t-test : * p<0.05 ; *** p<0.005 (first vs. second exposure)*

Conclusion

Unlike diazepam, α-casozepine when administered intraperitoneally at the doses of 0.4 and 0.8 mg/kg, did not show any amnesic effect on social memory in male Wistar rats.

Tolerance to the anxiolytic effect of Lactium®

Test aim

The study aimed at assessing the phenomenon of tolerance to Lactium® in male Wistar rats after chronic administration, using the conditioned defensive burying test.

Treatment

Lactium® was dissolved in 0.9% NaCl solution and administered twice daily i.p. at a dose of 12 mg/kg body weight for 4 days. On day 5, the product was administered at a dose of 6 mg/kg body weight, i.p., 30 minutes before testing (Appendix 1). Diazepam was used as positive control. It was dissolved in 0.9% NaCl solution and i.p. administered twice daily at a dose of 2 mg/kg body weight for 4 days. On day 5, it was administered at a dose of 1 mg/kg body weight, i.p. 30 minutes before testing. The vehicle (NaCl) was used as negative control.

Results

After repeated administration, Lactium® at a dose of 6 mg/kg body weight significantly decreased the global anxiety score while diazepam at a concentration of 1 mg/kg body weight did not (Fig. 21).

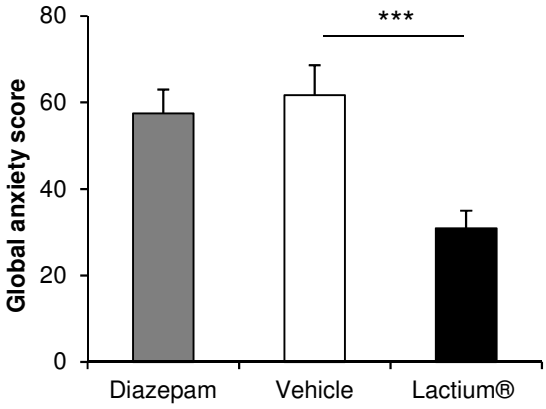


Figure 22. G*lobal anxiety score of rats after repeated administration of Lactium® and Diazepam. Data are mean ± SEM. Unpaired t-test (2-tail.) : *** p<0.005 (vs vehicle)*

Conclusion

Comparable to a single administration, Lactium® still lowered the global anxiety score after 4 days of pre-treatment. In contrast, tolerance to diazepam was observed after four days of pre-treatment in the conditioned defensive burying model in male Wistar rats.

Potential disinhibiting effect of Lactium®

Test aim

The study aimed at evaluating the possible disinhibiting effect of Lactium® in male Wistar rats using the conditioned defensive burying model.

Treatment

Sixty minutes before the conditioned defensive burying test sessions (Appendix 1), the rats received either 15 mg/kg body weight of Lactium®, 3 mg/kg body weight of diazepam (positive control), or vehicle (methyl-cellulose).

Results

The global anxiety score was significantly lower for Lactium® and diazepam when compared to placebo (Fig. 22). The latency of the first contact with the probe after the initial electrical shock is lower in the diazepam group compared to the placebo one (Fig. 23). This latency time is longer for rats treated with Lactium®.

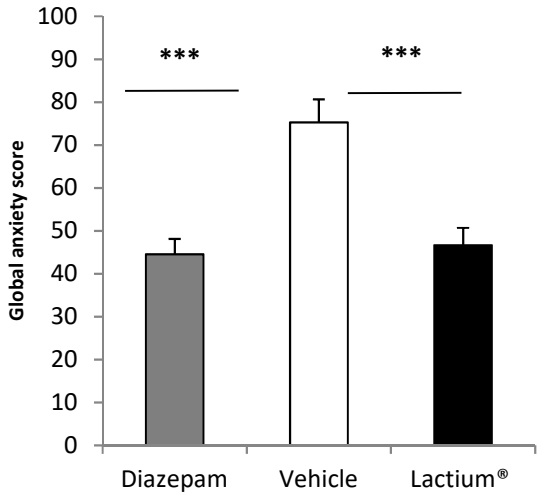


*Figure 23. Global anxiety score of rats. Data are mean ± SEM. Unpaired t-test (2-tail.): ** p<0.01 (vs. vehicle)*

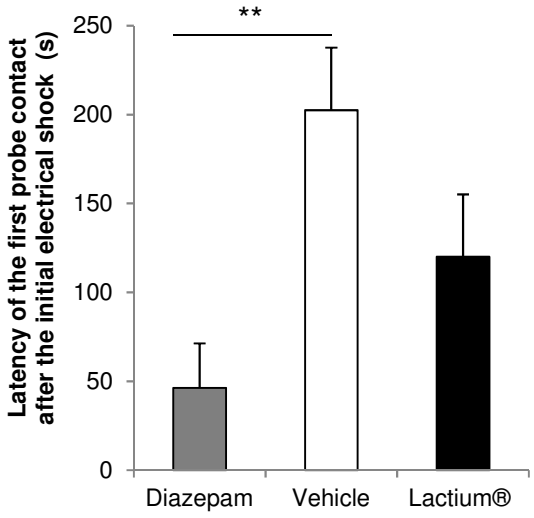


*Figure 24. Latency of the first contact with the probe after the initial electrical shock (s). Data are mean ± SEM. Unpaired t-test (2-tail.): *** p<0.001 (vs. vehicle)*

Conclusion

Unlike the rats treated with diazepam, which touch rapidly once more the aversive probe after the initial electrical shock, the rats receiving a single intake of 15 mg/kg body weight of Lactium® show less and non-significant disinhibited behaviour towards the electrical probe.

REFERENCES

Scientific publications on α-casozepine and Lactium®

Beata C., Beaumont-Graff E., Coll V., Cordel J., Marion M., Massal N., Marlois N., Tauzin J. *Effect of alpha-casozepine (Zylkene) on anxiety in cats*. J Vet Beh 2007a; 2:40-46.

Beata C., Beaumont-Graff E., Diaz C., Marion M., Massal N., Marlois N., Muller G., Lefranc C. *Effects of alpha-casozepine (Zylkene) versus selegiline hydrochloride (Selgian, Anipryl) on anxiety disorders in dogs.* J Vet Beh 2007b; 2:175-138.

Boulier A., Violle N. *Evaluation of the mechanism of Actium of Lactium®, a milk hydrolysate enriched in alpha-casozepine with anxiolytic properties*. NUTRITION 2018

Campell A., Neill, A.. *A randomized placebo controlled trial of melatonin enriched milk – can it improve symptoms of insomnia?* SLEEP 2015, Volume 38, Abstract Supplement. (Poster communication at the 29th Annual Meeting of the Associated Professional Sleep Societies, Seattle, United States)

Dela Peña I.J., Kim H.J., de la Peña J.B., Kim M., Botanas C.J., You K.Y., Woo T., Lee Y.S., Jung J.C., Kim K.M., Cheong JH. *A tryptic hydrolysate from bovine milk αs1-casein enhances pentobarbital-induced sleep in mice via the GABAA receptor.* Behav Brain Res. 2016 Jul 9;313:184-190.

De Saint Hilaire Z., Messaoudi M., Desor D., Kobayashi T. *Effects of a bovine αs1-casein tryptic hydrolysate (CTH) on sleep disorder in Japanese general population.* Open Sleep Journal 2009, 2; 26-32.

Guesdon B., Messaoudi M., Lefranc-Millot C., Fromentin G., Tomé D., Even P.C. *A tryptic hydrolysate from bovine milk αs1-casein improves sleep in rats subjected to chronic mild stress.* Peptides 2005, 27 (6):1476-82.

Jacquet A., Grolleau A., Jove J., Lassalle R., Moore N. *Burnout: Evaluation of the efficacy and tolerability of Target 1® for professional fatigue syndrome (burnout).* J Int Med Res. 2015 Feb; 43(1):54-66.

Kim J.H., Desor D., Kim Y.T., Yoon W.J., Kim K.S., Jun J.S., Pyun K.H., Shim I. *Efficacy of αs1-casein hydrolysate on stress-related symptoms in women.* Eur J Clin Nutr. 2007; 61 (4):536-41.

Kim J.H., Kim J., Lee S., Kim B., Kwon E., Lee J.E., Chun M.Y., Lee C.Y., Boulier A., Oh S., Lee H.W., *A Double-Blind, Randomized, Placebo-Controlled Crossover Clinical Study of the Effects of Alpha-S1 Casein Hydrolysate on Sleep Disturbance*. Nutrients. 2019; 11, 1466

Lanoir D., Canini F., Messaoudi M., Lefranc C., Demagny B., Martin S. & Bourdon L. *Long term effects of a bovine milk αs1-casein hydrolysate on healthy low and high stress responders.* Stress 2002, 5 (suppl.), 124 (Poster communication at the 4th World Congress on Stress in Edinburgh, UK).

Lecouvey M., Frochot C., Miclo L., Orlewski P., Driou A., Linden G., Gaillard J.-L., Marraud M., Cung M. T. & Vanderesse R. *Two-dimensional H-NMR and CD structural analysis in a micellar medium of a bovine αs1-casein fragment having benzodiazepine-like properties.* Eur. J. Biochem. 1997a, 248 (3), 872-878.

Lecouvey M., Frochot C., Miclo L., Orlewski P., Marraud M., Gaillard J.-L., Cung M. T. & Vanderesse R. *Conformational studies of a benzodiazepine-like peptide in SDS micelles by circular dichroism, H NMR and molecular dynamics simulation.* Lett. Pept. Sci.1997b, 4, 359-364.

Messaoudi M., Bresson J.-L., Desor D., Lefranc C., Boudier J.-F. & Paquin P. *Anxiolytic-like effects of the milk protein hydrolysate Lactium® in healthy human volunteers.* Stress 2002, 5 (suppl.), 124 (Poster communication at the 4th World Congress on Stress in Edinburgh, UK).

Messaoudi M., Lefranc-Millot C., Desor D., Demagny B., Bourdon L. *Effect of a tryptic hydrolysate from bovine milk αs1-casein on hemodynamic responses in healthy human volunteers facing successive mental and physical stress situations.* Eur J Nutr 2004, 44 (2):128-32.

Miclo L., Perrin E., Driou A., Papadopoulos V., Boujrad N., Vanderesse R., Boudier J.-F., Desor D., Linden D. & Gaillard J.-L. *Characterization of α-casozepine, a tryptic peptide from bovine α_{S1}-casein with benzodiazepine-like activity.* FASEB J. 2001; 15(10):1780-2.

Violle N., Messaoudi M., Lefranc-Millot C., Desor D., Nejdi A., Demagny B., Schroeder H. *Ethological comparison of the effects of a bovine αs1-casein tryptic hydrolysate and diazepam on the behaviour of rats in two models of anxiety.* Pharmacol Biochem Behav 2006, 84 (3), 517-523.

List of patents

Miclo L., Perrin E., Driou A., Boudier J.F., Lung C. & Linden G. (1995). *Use of a decapeptide with a benzodiazepine activity for the preparation of medicaments and dietary supplements.* EP patent number 0714910B1.

Miclo L., Perrin E., Driou A., Boudier J.F., Lung C. & Linden G. (1998). *Use of a decapeptide with benzodiazepin activity for preparing medicines and food supplements.* US patent number 5,846,939.

Other references

Buysse D.J., Reynolds III C.F., Monk T.H., Berman S.R. & Kupfer D.J. *The Pittsburgh Sleep Quality Index: A New Instrument for Psychiatric Practice and Research.* Journal of Psychiatric Research 1989, 28(2), 193-213.

Craft R.M., Howard J.L. & Pollard G.T. *Conditioned defensive burying as a model for identifying anxiolytics.* Pharmacol. Biochem. Behav. 1998, 30, 3, 775-780.

Cruz A. P. M., Frei F. & Graef F. G. *Ethopharmacological analysis of rat behavior on the elevated plus maze.* Pharmacol. Biochem. Behav. 1994, 49, 171-176.

De Boer S.F. & Koolhaas J.M. *Defensive burying in rodents: ethology, neurobiology and psychopharmacology.* European Journal of Pharmacology 2003, 463, 145– 161.

Freyschuss U., Fagius J., Wallin B., Bohlin G., Perski A. & Hjemdahl P. *Cardiovascular and sympathoadreanal responses to mental stress: a study of sensory intake and rejection reactions.* Acta Physiol Scand. 1990, 139, 173-183.

Johns M.W. *A new method for measuring daytime sleepiness: the Epworth sleepiness scale.* Sleep 1991; 14 (6), 540 – 545.

Lister R. G. *The use of a plus maze to measure anxiety in the mouse.* Psychopharmacology 1987, 92, 180-185.

Milagres M.P., Minim VP, Minim LA, Simiqueli AA, Moraes LE, Martino HS. *Night milking adds value to cow's milk.* J Sci Food Agric. 2014 Jun;94(8):1688-92.

Pellow S., Chopin P., File S. & Briley M. *Validation of open: close arm entries in the elevated plus maze as a measure of anxiety in the rat.* J. Neurosci. Methods 1985, 14, 149-167.

Pinel J.P. & Treit D. *Burying as a defensive response in rats.* J. Comp. & Physiol. Psychol. 1978, 92, 708-712.

Prus A.J., James J.R. & Rosecrans J.A. Chapter 4: *Conditioned Place Preference.* In: Methods of Behavior Analysis in Neuroscience 2009. 2nd edition.

Rodgers R. J. & Johnson N. J. T. *Factor analysis of spatiotemporal and ethological measures in the murine elevated plus-maze test of anxiety.* Pharmacol Biochem Behav. 1995;52(2):297-303.

Stroop J. R. *Studies of interference on serial verbal reactions.* J. Exp. Physiol. 1935, 18 : 643-662

Thor, D. H. & Holloway, W. R. *Social memory of the male laboratory rat.* J. Comp. Physiol. Psychol. 1982, 96: 1000-1006.

Treit D., Pinel J.P. & Fibiger H.C. *Conditioned defensive burying: a new paradigm for the study of anxiolytic agents.* Pharmacol. Biochem. Behav. 1981, 15, 619-626.

Weise F., Laude D., Girard A., Zitoun P., Siché J.P. & Elghozi J.L. *Effects of the cold pressor test on short-term fluctuations of finger arterial blood pressure and heart rate in normal subjects.* Clin. Auton. Res. 1993, 3, 303-310.

APPENDICES

APPENDIX 1: Conditioned Defensive Burying Test

APPENDIX 2: Elevated Plus Maze

APPENDIX 3: Stroop and Cold Pressor Tests

APPENDIX 4: Conditioned Place Preference Test

APPENDIX 5: Social Memory Test

APPENDIX 1: Conditioned defensive burying test

The conditioned defensive burying (CDB) test is based on a procedure developed by Treit *et al.* (1981). Defensive burying is a typical behaviour of rodent to displace bedding material towards a noxious stimulus that poses a near and immediate threat, such as an electrified shock-probe (De Boer and Koolhaas, 2003).

For a rat, burying is part of its natural behavioural repertoire; it requires no training and is elicited by aversive stimulation. Anxiolytic agents decrease the time of burying and avoidance behaviour expressed by rodents in these situations. Analysis of this behaviour is substituted by other behavioural measures connected to anxiety, such as the number of head stretches towards the probe, and the sequence of approaches towards and retreats from the probe.

As anxiolytics decreased or suppressed burying, it was suggested that the CDB test is a suitable preclinical screening method for anti-anxiety agents (Craft *et al.,* 1998).

Test principle

Male Wistar rats are placed in a cage with the floor covered by 5 cm of bedding material. On the opposite side of the cage, an electric shock-probe is installed which will be buried with bedding material by the rat. A camera records the behaviour without the presence of an investigator in the experimental room.

Anxiety test

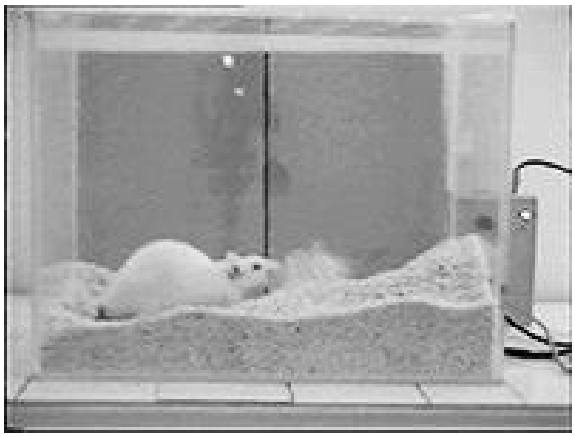
For habituation to the test conditions, each rat is placed in the test chamber without shock-probe for 20 minutes the two days prior to the test. The test is performed during the first hours of the dark cycle; the period during which the rats are the most active. The shock-probe is inserted into the chamber before the test session. The rat is placed in the test chamber on the side opposite the shock-probe. The first time the rat touches the probe with its forepaws, the experimenter delivers a single shock of mild intensity. Immediately after the shock administration, the behaviour of each rat is recorded for 5 minutes.

Variables studied and calculation of the global anxiety score

The following behaviour is quantified based on the videos registered during the test:

- Duration of probe-burying
- Number of head stretches towards the probe
- Number of approaches towards the probe
- Number of retreats away from the probe

The percentage of approaches towards the probe followed by retreats is calculated as followed: (number of approaches/number of retreats) * 100. In order to obtain the global anxiety score, values of the variables “duration of probe-burying”, “number of head stretches” and “percentage of approaches followed by retreats” are classified in increasing order and then transformed in their respective ranks. For each rat, the sum of these ranks represents its anxiety global score which can subsequently be compared.



Rat during the Conditioned Defensive Burying Test

References

Craft RM, Howard JL, Pollard GT. *Conditioned defensive burying as a model for identifying anxiolytics.* Pharmacol Biochem Behav. 1988 Jul;30(3):775-80.

De Boer SF, Koolhaas JM. *Defensive burying in rodents: ethology, neurobiology and psychopharmacology.* Eur J Pharmacol. 2003 Feb 28;463(1-3):145-61. Review.

Treit D, Pinel JP, Fibiger HC. *Conditioned defensive burying: a new paradigm for the study of anxiolytic agents.* Pharmacol Biochem Behav. 1981 Oct;15(4):619-26.

APPENDIX 2: Elevated plus maze

The elevated plus maze (EPM) is a widely used animal model of anxiety in behavioural pharmacology. It is based on the natural aversion of rodents for open space, negatively loaded on anxiety level of the animal (Cruz *et al.*, 1994, Rodgers & Johnson, 1995). The test was validated for rats (Pellow *et al.*, 1985) and mice (Lister, 1987).

Test principle

Rats are placed in an apparatus consisting of two open arms and two enclosed arms that extend from a central platform. Their movements and time spent in the two types of arms is recorded. It is suggested that the total number of entries is related to locomotor activity and the percentage of time spent on the central platform is related to decision-making and risk assessment (Cruz *et al.* 1994, Rodgers & Johnson, 1995).

Anxiety test

The test is performed during the first hours of the dark cycle; the period during which the rats are the most active. The test maze with its four arms is elevated to a height of 70 cm above floor level. The protected area of the maze is composed of the closed arms and the central platform.

The rat is placed in the centre of the apparatus, head turned towards an open arm. It is then left to move freely for 5 minutes, during which its behaviour is recorded by a camera.

Variables studied

The following behaviour is quantified based on the videos registered during the test:

- Number of open and closed arms entries, total number of entries
- Amount of time spent in open arms, closed arms and on the central platform
- Head dips

Elevated plus maze apparatus

References

Cruz AP, Frei F, Graeff FG. *Ethopharmacological analysis of rat behavior on the elevated plus-maze.* Pharmacol Biochem Behav. 1994 Sep;49(1):171-6.

Lister RG. *The use of a plus-maze to measure anxiety in the mouse.* Psychopharmacology (Berl). 1987;92(2):180-5.

Pellow S, Chopin P, File SE, Briley M. *Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat.* J Neurosci Methods. 1985 Aug;14(3):149-67.

Rodgers RJ, Johnson NJ. *Factor analysis of spatiotemporal and ethological measures in the murine elevated plus-maze test of anxiety.* Pharmacol Biochem Behav. 1995 Oct;52(2):297-303.

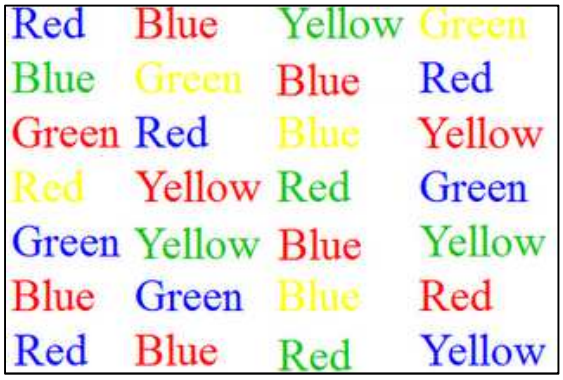
APPENDIX 3: Stroop test and Cold Pressor tests

In order to induce a stressful test situation for the evaluation of the effect of Lactium®, two different stimulations were used in the clinical trials. The Stroop test (Stroop, 1935) induces a psychological stress while the Cold pressor test induces a physical stress.

Stroop colour-word interference test

The Stroop colour-word interference test is originally used for neuropsychological assessment. The task is to name colours presented either in black ink or in coloured ink while during the latter, the colour of the word and the ink can be different. The test assesses attention and cognitive capacity but the colour conflict also induces mental stress and triggers hypertension (Freyschuss *et al.*, 1990).

In our studies a simplified version of the Stroop test was used, while recording their blood pressure and heart rate. A series of four colours written with words in a different colour was presented on a computer screen. Participants were asked to identify the colours by pressing one of four keys mapped to the colours red, blue, green and yellow, on a computer keyboard using the index of the right hand. Any errors were signalled with a bell.

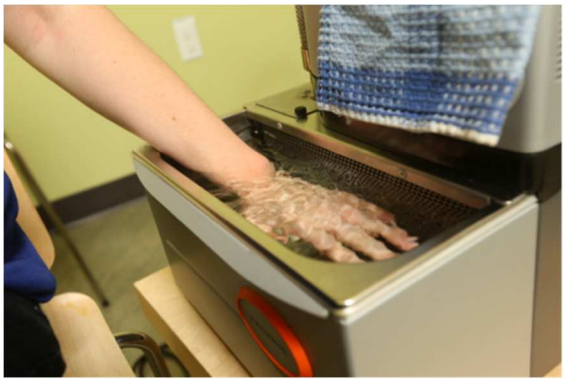


Example of stimuli and colours used for the Stroop test.

Cold pressor test

The cold pressor test is commonly used to investigate cardiovascular reactions such as blood pressure and heart rate in response to an environmental stress (Weise *et al.*, 1993).

Volunteers immersed their left hand for 5 minutes in slushy ice water (2 °C) past the wrist and slowly rotated the hand to maintain maximal cold stimulus. The immersion of the hand in cold water would product a cutaneous heat-sensory stimulation equivalent to a pain, which would cause a sympathetic nervous activation, as with any other stressing agent.



Hand of volunteer immersed in slushy ice water.

References

Freyschuss U., Fagius J., Wallin B., Bohlin G., Perski A. & Hjemdahl P. *Cardiovascular and sympathoadreanal responses to mental stress: a study of sensory intake and rejection reactions.* Acta Physiol Scand. 1990, 139, 173-183.

Stroop J. R. *Studies of interference on serial verbal reactions.* J. Exp. Physiol. 1935, 18 : 643-662.

Weise F., Laude D., Girard A., Zitoun P., Siché J.P. & Elghozi J.L. *Effects of the cold pressor test on short-term fluctuations of finger arterial blood pressure and heart rate in normal subjects.* Clin. Auton. Res. 1993, 3, 303-310.

APPENDIX 4: Conditioned Place Preference Test

The conditioned place preference paradigm is a standard preclinical behavioural model used to study the rewarding and aversive effects of drugs. In the test, the rat associates an "internal state of well-being" with a distinctive environment, the subsequent selection of which is considered to reflect the appetizing properties of the drug. After a certain number of administrations of an addicting compound, followed by a forced stay in the compartment initially non-preferred, the addicted animal will preferentially choose this compartment (Prus *et al.*, 2009).

Opiates, such as morphine, psychostimulants, such as amphetamine and minor tranquilizers, such as diazepam have been shown to induce conditioned place preference.

Test principle

The experimental apparatus consists of a rectangular box divided into two compartments separated by a guillotine door. The compartments are characterized by the colour of their walls and the texture of their floors: black walls with smooth floor vs. grey walls with corrugated floor.

- *Initial Preference*: This session was carried out over 3 days, 30 minutes per day, in order to familiarize the rats with the apparatus. The animal was allowed to move freely between compartments. On the third day each rat's preference for one of the two compartments was determined (compartment where the rat spent the most time).

- *Conditioning session*: On days 4, 6, 8 and 10, rats were treated with the tested compound and individually enclosed in their initially non-preferred compartment for 45 minutes.

On alternate days, each rat received a vehicle injection and was individually enclosed in the initially preferred compartment for 45 minutes.

- *Preference testing*: On the 12th day, the addiction test was carried out: the rats were individually placed between the compartments for 30 minutes with free access to both compartments. The time spent in each compartment was measured.

Variables studied

- Time spent in each compartment on the 3rd day (determination of the initially preferred compartment).

- Time spent in each compartment during preference testing (used as an indicator of addiction).

Conditioned place preference apparatus

Different floor textures in conditioned place preference

References

Prus A.J., James J.R. & Rosecrans J.A. Chapter 4: *Conditioned Place Preference.* In: Methods of Behavior Analysis in Neuroscience 2009. 2nd edition.

APPENDIX 5: Social Memory Test

The social memory test proposed by Thor & Holloway in 1982 is based on the tendency of rodents to investigate any unfamiliar conspecific. This investigation consists mainly in sniffing, nosing, following and grooming. The use of juvenile conspecific as social stimulus avoids aggression and sexual behaviour of the tested rat. When a juvenile is presented for the first time to the tested rat, it is intensely investigated. When it is presented for a second time shortly after, it triggers much less attention. Elderly rats or those treated with benzodiazepines or vasopressin antagonists just after the first exposure, presented with the same juvenile 30 minutes after the initial exposure, behave towards this social stimulus as if it was no longer recognized, meaning they spent as long investigating the juvenile as on the occasion of the first exposure.

Test principle

The rat was placed in the experimental room and remained there for 2 hours prior to the first exposure. Each test consisted of a 5 minute exposure to a juvenile, followed by a second 5 minute exposure to the same juvenile 30 minutes later. Between the two successive presentations, juveniles were kept individually in small boxes.

The test products were intraperitoneally injected to rats immediately after the first exposure to the stimulus juvenile. Investigations were defined as direct contact (nosing, sniffing) of the adult rat with the ano-genital areas of the juvenile.

Variables studied

The studied variable was the duration of the investigation of the juvenile by the adult rat during the first minute and during the 5 minute period of each test.

Reference

Thor, D. H. & Holloway, W. R. *Social memory of the male laboratory rat.* J. Comp. Physiol. Psychol. 1982, 96: 1000-1006.