Effects of lidocaine on torn rotator cuff tendons

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ABSTRACT

We determined lidocaine's action on torn rotator cuff tendons *in vitro* and *in vivo*. For *in vitro* experiments, cell proliferation and viability assays were performed using tenocytes derived from human torn rotator cuff tendons. For *in vivo* experiments, acute rotator cuff tears were made on the supraspinatus tendons in the rats' bilateral shoulders; before closure, lidocaine was injected into the shoulder and saline into the contralateral shoulder (control). After sacrifice, the specimens underwent biomechanical testing or histological analysis at 24 h and at 2, 4, and 8 weeks after surgery. The extent of collagen organization and apoptosis were semi-quantitatively evaluated using collagen picrosirius red staining. Apoptosis was examined using TUNEL staining and electron microscopy. Cell proliferation decreased dose-dependently. After exposure to 0.1% lidocaine for 24 h, cell viability decreased. Two and 4 weeks after surgery, the ultimate load to failure decreased more in the lidocaine group than in the control group, with significantly reduced stiffness in the lidocaine group 2 weeks after surgery. Collagen organization

significantly decreased in the lidocaine group by 4 weeks after surgery but returned to baseline at 8 weeks. TUNEL staining detected numerous apoptotic tenocytes at the torn tendon edge exposed to lidocaine 24 h after surgery; electron microscopy confirmed the condensed cell nuclei. These changes were not observed in controls. Lidocaine caused cytotoxicity to tenocytes under both conditions, decreased biomechanical properties, and induced apoptosis and delay of collagen organization in this model. Subacromial lidocaine injections in patients with rotator cuff tears should be performed carefully.

Keywords: lidocaine, rotator cuff, tendons, tenocytes

Introduction

Local anesthetics are clinically used for the treatment of tendinopathies. Peritendinous injections of local anesthetics, in combination with other drugs (e.g., steroids), are given around various joints to treat lateral and medial epicondylitis of the elbow, de Quervain's disease, patellar and pes anserine tendinopathies, and Achilles tendinopathy [1-6]. Similarly, subacromial injections of anesthetic agents are used in rotator cuff tears as therapeutic and diagnostic tools [7].

However, *in vitro* studies have shown the toxicity of local anesthetics on tendon fibroblasts [8-11]. Lehner et al. reported that the treatment of rat tendon-derived cells with 0.5% bupivacaine for 10 min had detrimental effects on cell viability [8]. Piper et al. showed a toxic effect of 30 min of 1% lidocaine treatment on bovine tendon fibroblasts [9]. Yang et al. concluded that lidocaine potentiated the deleterious effects of triamcinolone acetonide on cultured tenocytes derived from rat-patellar tendons [10]. Recently, Sung et al. compared the cytotoxic effects of ropivacaine, bupivacaine, and lidocaine on human rotator cuff tendon fibroblasts and showed high cytotoxicity of lidocaine compared with the other agents, even in low concentration [11].

Despite the deleterious effects of the local anesthetics as evaluated by *in vitro* studies [8-11], lidocaine is frequently used preoperatively in clinical practice in rotator cuff tears [12-15];

however, *in vivo* data on the effects of lidocaine are lacking. These results prompted us to examine how lidocaine acts on rotator cuff tears when used in subacromial injection for this disease. Thus, the purpose of the present study was to evaluate the effects of lidocaine on cultured tenocytes from human rotator cuff tendons in an animal cuff tear model. We hypothesized that lidocaine has deleterious effects on tenocytes *in vitro* and *in vivo*.

Methods

Lidocaine was purchased from Maruishi (1% lidocaine injection; Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM) and L-glutamine were purchased from Nissui Seiyaku (Tokyo, Japan), and fetal bovine serum (FBS) was purchased from Thermo Scientific (Tokyo, Japan).

Study Design

All applicable international, national, and our institutional guidelines for the care and use of animals and Ethics Committee were followed. Informed consent was obtained from all individual participants included in the study. All tests and measurements were performed blindly.

For the *in vitro* experiments, the tenocytes obtained from torn human rotator cuff

tendons were cultured in monolayers at various concentrations of lidocaine (0.001%, 0.01%, 0.05%, and 0.1%); 9 specimens were used in the cell-proliferation assay, and 5 specimens were used in the cell-viability assay. A singlicate analysis was performed to collect experiment data.

For the cell-proliferation assay, cultured cells from a specimen were divided into 5 groups and exposed to 5 graded concentrations of lidocaine (control, 0.001%, 0.01%, 0.05%, and 0.1%). Therefore, 9 data points were obtained in total. For the cell-viability assay, cultured cells from a specimen were divided into 2 groups and exposed to either 0% or 1% lidocaine. Therefore, 5 data points were obtained in total.

For the *in vivo* experiments, adult Sprague–Dawley rats (n = 33) underwent bilateral shoulder surgery, and a total of 66 shoulders were assessed. The shoulders were allocated into 2 groups: lidocaine and control. Each group consisted of 6 specimens for biomechanical testing and 3 for histological analysis and was evaluated 2, 4, and 8 weeks after surgery. Six specimens in each group were subjected to 3 TUNEL staining and 3 electron microscopy analyses 24 h after surgery (Figure 1).

Tissue preparation

Fourteen patients (9 males, 5 females) with rotator cuff disease were included in this study. Nine subjects had right shoulder involvement, and 5 subjects had left shoulder

involvement. There were 2 small, 5 middle, 3 large, and 4 massive tears. Average subject age was 62.4 ± 9.6 years, and the average period from symptom onset was 69.7 ± 73.8 weeks. All the patients had received subacromial injections of a steroid or hyaluronic acid in addition to physical therapy. Arthroscopic cuff repair was performed for a minimum of 2 months after the last subacromial injection to minimize any potential effects of the agents.

The specimensSpecimens of the rotator cuff tendon were 3–5 mm in width and were obtained from the margins of human rotator cuff tendons. The biopsy was performed in the supraspinatus tendon and partly in the infraspinatus tendon when the tear size was large or massive. Care was taken not to contaminate other types of adjacent tissue (muscle or synovium). The cuff tear was completely repaired without excessive tension at the sides.

Tenocytes Culture

Torn human rotator cuff tendons were obtained from 14 patients during surgery, and explant cultures were performed to isolate tenocytes. The tissues were rinsed twice in phosphate-buffered saline (PBS) and cut into small pieces, and the tenocytes were maintained in culture medium consisting of high-glucose DMEM (Nissui, Tokyo, Japan), 10% FBS (Thermo Scientific, Utah, USA), and 1% penicillin/streptomycin (Nacalai Tesque, Kyoto, Japan) in an incubator at 37°C with 5% CO_2. The culture medium was changed twice a week. The tenocytes

120 from the second passage were used for the experiments.

121

122 **Cell Proliferation Assay**

123 Cultured human tenocytes were seeded onto 96-well plates at a density of 5,000
124 cells/well and incubated with DMEM containing 10% FBS for 24 h. The tenocytes were treated
125 with lidocaine (Maruishi, Osaka, Japan) at various concentrations (0.001%, 0.01%, 0.05%, and
126 0.1%) for the next 24 h. After a 3-h reaction with WST-8 cell count reagent (Nacalai Tesque), the
127 fluorescence intensity of each well was measured using a microplate reader (BIO-RAD Model
128 550; Bio-Rad Laboratories, Richmond, CA, USA) at a wavelength of 450 nm.

129

130 **Cell Viability Assay**

131 Cultured human tenocytes were seeded onto 6-well plates at a density of 1.5×10^5
132 cells/well and incubated with DMEM containing 10% FBS for 24 h. The cells were exposed to
133 0.1% lidocaine or only to medium (controls) for the next 24 h. Floating cells and trypsinized
134 adherent cells were combined and diluted to 1×10^6 cells/ml with 1X PBS. The components of a
135 LIVE/DEAD Cell Viability Assay Kit (Invitrogen, OR, USA), C12-Resazurin, and SYTOX
136 GREEN were applied to stain viable and dead cells. After incubation for 15 min at 37°C, the
137 numbers of viable and dead cells were obtained by using a FACS instrument (BD Bioscience,

USA).

Rotator Cuff Tear Model in Rats

Thirty-three adult Sprague–Dawley male rats (mean body weight, 487.4 ± 93.2 g) were used according to the guidelines of the Institutional Animal Care and Use Committee. Animals were anesthetized with isoflurane under a high flow rate of oxygen. A middle skin incision was made and subcutaneous tissue was divided. After the deltoid was divided to expose the subacromial space, a complete tear was made on the supraspinatus (SSp) tendon with a #11 scalpel blade. The supraspinatus tendon was totally resected with preservation of its cartilaginous portion and adjacent tendons (the infraspinatus and subscapularis tendons). Then, the myotendinous unit was immediately retracted (Figures 2a, b). Before skin closure, 0.1 μl (1% lidocaine)/body weight (g), dose used in the rat is equal to 0.1 ml/1% lidocaine/body weight (kg) in humans, was injected onto the tendon around the deficit site, and PBS was injected onto the contralateral shoulder as a control. The animals were allowed to move freely in their cages after surgery.

Biomechanical Testing

All specimens were immediately tested after sacrifice. Soft tissues were removed except for the SSp tendon–humerus complex. Each specimen was then placed into a uniaxial testing machine (TENSILON RTE-1210; Orientec, USA). The SSp tendon was secured in a screw grip by using sandpaper and ethyl cyanoacrylate, and the humerus was secured in a custom-designed pod by using a capping compound. The SSp tendon–humerus complex was positioned to allow the tensile loading in the longitudinal direction of the injured site for the SSp tendon (Figure 2b). The specimens were preloaded at 0.1 N for 5 min, followed by 5 cycles of loading and unloading with 0.5 N at a cross-head speed of 5 mm/min and then loaded until failure at 1 mm/min. The ultimate load to failure and linear stiffness were calculated from the resulting load-elongation curve. This testing protocol was similar to that described previously [16, 17].

Hematoxylin and Eosin (HE), Picrosirius Red, and Terminal Deoxynucleotidyl Transferase dUTP-mediated Nick End-labeling (TUNEL) Staining

The SSp tendon–humerus complex was fixed in 10% buffered formalin and then decalcified with formic acid solution. Tissues were processed for paraffin embedding. Longitudinal sections of 5-μm thickness of the SSp tendon–bone complex were placed on the glass slides and stained with either HE or picrosirius red. The specimens were visualized under a light and polarized light microscope (OLYMPUS BX50; OLYMPUS. Tokyo Japan), and

174 photomicrographs were obtained using an Olympus DP71 digital camera (Olympus).

175

176 Picrosirius red staining was used for the evaluations of the collagen organization [17-
177 20]. For the semi-quantitative analysis, 2 of 3 glass slides were randomly selected from each
178 group, and photomicrographs were taken at 100× magnification. Consequently, a total of 36
179 photomicrographs were digitized (8-bit) using ImageJ software at a resolution of 1360 × 1024
180 pixels. In each photomicrograph, 10 randomly selected areas (50 μm × 50 μm) were evaluated by
181 measuring the degree of gray scale on the area, yielding an image in which non-collagenous
182 tissue was dark, and collagen was depicted on the gray scale of 1–255. Finally, the average
183 values of 10 areas in each specimen were calculated; higher values indicated more organized
184 collagen. This testing protocol was similar to that described previously [19, 20].

185 To evaluate apoptosis at the torn site, tendon specimens were subjected to TUNEL
186 staining 24 h after surgery using an *in situ* cell death kit (WAKO Chemical, Japan) according to
187 the manufacturer's instructions. DNase-digested sections served as a positive control, and TdT
188 (−) sections served as a negative control.

189

190 **Ultrastructure Analysis by Low-Voltage Scanning Electron Microscopy (LV-SEM)**

191 Tenocyte morphology was analyzed 24 h after surgery using wide-range observation of
192 the flat block face by LV-SEM.
193 Sprague–Dawley rats were deeply anesthetized, transcardially perfused through the left
194 ventricle with heparin-containing saline, and subsequently fixed with half Karnovsky's solution
195 (2% paraformaldehyde, 2.5% glutaraldehyde, and 2 mM $CaCl_2$ in 0.1 M cacodylate buffer). After
196 perfusion, the SSp tendon–humerus complex was harvested and immersed in the same fixative
197 for 2 h at 4°C. After decalcification with Kalkitox solution (WAKO Chemical), the specimens
198 were cut into small cubes and fixed with ferrocyanate and 1% OsO_4, treated with 1%
199 thiocarbohydrazide, and then immersed in 1% OsO_4. For en bloc staining, the specimens were
200 immersed overnight in a solution of 4% uranyl acetate and washed with distilled water. Next, the
201 specimens were stained with Walton's lead aspartate solution, dehydrated with a graded ethanol
202 series, infiltrated with an epoxy resin mixture, and polymerized at 60°C for 72 h. The resin
203 blocks containing the entire surgical site were trimmed to 2 mm × 4 mm in size, and the surfaces
204 of the embedded specimens were exposed using a diamond knife. Ultrastructural
205 photomicrographs of various regions at the surgical sites, as material contrast images resembling
206 transmission electron microscopy images referred to as block face images [19], were obtained
207 from the block surface of the same specimen using LV-SEM. The images were obtained under
208 the following conditions: accelerating voltage = 2 kV, dwell time = 30 μ seconds, and image size

209 = 2048 × 1768.

210

211 **Statistical Analysis**

212 Statistical analysis was performed by using JMP version 11 (SAS Institute Inc., Cary,

213 NC, USA). Kruskal–Wallis test with the Wilcoxon test as a *post-hoc* analysis was used to

214 evaluate lidocaine and control groups by comparing cell proliferation and viability. Two-way

215 ANOVA test of variance with the Wilcoxon test as a *post-hoc* analysis was used to evaluate

216 biomechanical data and picrosirius red staining (gray scale). Values were shown as mean ±

217 standard deviation (SD). Differences with a P value <0.05 were considered to be significant.

218

219 **Results**

220 **Cell Proliferation**

221 When the values in the control group were defined as 100%, the relative ratio

222 (lidocaine/control) was 97.7% for 0.001% lidocaine, 95.6% for 0.01% lidocaine, 85.2% for

223 0.05% lidocaine, and 68.2% for 0.1% lidocaine (Figure 3a). Thus, lidocaine significantly

224 decreased cell proliferation of the cultured human tenocytes in a dose-dependent manner (P <

225 0.05).

226

227 **Cell Viability**

228 Under exposure to 0.1% lidocaine for 24 h, the FACS quantification analysis showed
229 significantly decreased cell viability in the lidocaine groups (% live cells, 75.0 ± 7.0) relative to
230 that in the control groups (% live cells: 86.9 ± 8.0) (Figure 3b).

231

232 **Biomechanical Strength**

233 All specimens tested failed at the site of the SSp tendon tear. The ultimate load to failure
234 values (lidocaine vs. control group) were 11.5 ± 3.5 N and 21.4 ± 2.5 N at 2 weeks after surgery
235 ($P < 0.05$), 17.5 ± 3.1 N and 22.2 ± 2.1 N at 4 weeks after surgery ($P < 0.05$), and 23.3 ± 4.0 N
236 and 29.8 ± 8.1 N ($P = 0.13$) at 8 weeks after surgery (Figure 4a). Thus, significant differences
237 between the 2 groups were noted at 2 and 4 weeks after surgery.

238 The stiffness values (lidocaine vs. control group) were as follows: 9.9 ± 6.1 N/mm and
239 19.6 ± 6.3 N/mm at 2 weeks after surgery ($P < 0.05$), 15.5 ± 4.0 N and 18.0 ± 2.6 N at 4 weeks
240 after surgery ($P = 0.3$), and 27.1 ± 8.2 N and 30.8 ± 12.1 N at 8 weeks after surgery ($P = 0.42$)
241 (Figure 4b). Thus, significant differences between the 2 groups were noted only at 2 weeks after
242 surgery.

243

244 **Evaluation of Fibrovascular Tissue at the Torn Site**

Two weeks after surgery, the abundant fibro-vascular tissue from the extra-articular side encroached on the torn tendons in both groups. Compared with the control group, the thickness of the fibro-vascular tissue was relatively lesser in the lidocaine group, with irregular collagen bundles and less cellularity. These changes continued till 4 weeks after surgery, but no apparent difference was seen 8 weeks after surgery.

Evaluation of the Collagen Organization at the Torn Site

Extent of the collagen organization at the torn site (lidocaine vs. control) was significantly lesser in the lidocaine group: the gray scale values were 32.0 ± 19.0 and 49.9 ± 27.9 at 2 weeks after surgery ($P < 0.05$) and 41.0 ± 15.1 and 57.7 ± 22.9 at 4 weeks after surgery ($P < 0.05$); however, there was no significant difference in the values at 8 weeks after surgery (lidocaine vs. control: gray scale values of 66.3 ± 25.5 and 68.4 ± 27.4) ($P = 0.82$) (Figures 5a, 5b, 5c).

Apoptotic Cells at the Torn Site

In the tendon specimens 24 h after surgery, a large number of TUNEL-positive cells were observed in the lidocaine group (Figure 6a) but not in the control group (Figure 6b).

Apoptotic Cells at the Torn Site Evaluated at the Ultrastructural Level

In the tendon specimens 24 h after surgery, segmentalized collagen fibrils and condensed nuclei of the tenocytes were observed at the edge of the torn tendon where lidocaine was directly injected (Figure 7a). In contrast, these findings were not evident in the control group. (Figure 7b)

Discussion

The delayed organization of collagen fiber in the rat rotator cuff tear model used in this study showed that lidocaine significantly inhibited cell proliferation and caused cell death in tenocytes from torn human rotator cuff and that lidocaine induced apoptosis, collagen necrosis, and decreased biomechanical strength at the tear site. Thus, this study showed that lidocaine caused adverse effects on tenocytes in torn rotator cuff tendons.

In vitro studies [21-24] have demonstrated considerable local anesthetic toxicity on various cell types, including tendon fibroblasts derived from bovine tendon [3] and torn human rotator cuff tendons [5]. Recently, an *in vitro* study revealed the cytotoxic mechanism of aminoamide local anesthetics acting on human rotator cuff tendon fibroblasts [5]. That study evaluated the response of tendon fibroblasts to ropivacaine, bupivacaine, and lidocaine and found that these anesthetics caused cell death that was mediated by increased production of reactive

281 oxygen species. The reactive oxygen species resulted from increased activation of extracellular
282 signal-regulated kinase 1/2, c-Jun N-terminal kinase, and p38 and by activation of caspase-3/7
283 [5]. Similarly, the *in vitro* experiments in the present study showed cytotoxic effects of lidocaine
284 on the tenocytes derived from human rotator cuff tendons.

285 Regarding the *in vivo* effects of local anesthetics on tendons, only a few reports have
286 addressed this topic. Lehner et al. showed that 0.5% bupivacaine elicited a temporary functional
287 damage after a single peritendinous injection on rat Achilles tendon and demonstrated cell
288 apoptosis at the injection site [2]. Friel et al. evaluated the effect of continuous subacromial
289 0.25% bupivacaine infusion on repaired SSp tendons in a rabbit rotator cuff repair model, and no
290 significant differences in the biomechanical and histological features were found between the
291 bupivacaine-treated and control groups [25]. In our rat cuff tear model, a single injection of 1.0%
292 lidocaine caused apoptotic changes in tenocytes 24 h after surgery and delayed organization at
293 the tear site by 4 weeks after surgery. These discrepancies appear to be either related to
294 differences in the animal models or the concentrations of local anesthetics used in these studies.

295 Unlike the “cuff tear” model in the present study or the “peritendinous injection” model
296 reported by Lehner et al. [2], a “tear and subsequent repair” model [26,27] may not have
297 predisposed tenocytes to be exposed at sufficient levels of concentration because the cells are
298 protected/covered by tendon repair. In contrast, the “cuff tear” model in the present study may

have caused relatively high-concentration exposure to the tenocytes, and this may also have occurred in the "peritendinous injection" model [2], in which the agents were potentially injected into the tendinous portion. Previous *in vitro* studies have demonstrated that local anesthetics inhibited cell proliferation and/or viability in a dose-dependent manner [4,23,28]. Thus, the milieu of the injection site seems to directly determine the effects of local anesthetics; in fact, in the present study, the tenocytes at the edge of the tear site that were directly exposed to lidocaine, but not the cells exposed to PBS (control), underwent apoptotic changes.

We previously demonstrated the healing capability of torn tendon edges in patients with rotator cuff tears by detecting procollagen types I and III gene expression in the tendon fibroblasts [29-32]. These procollagen gene expression levels at the edge of the torn tendon significantly correlated with re-tear after surgery [29]. In the present study, significant decrease in the ultimate load to failure and delay of collagen organization in the lidocaine group continued at 4 weeks after surgery. In a randomized, double-blind, prospective study, the accuracies of landmark- and ultrasound-guided injections were 70% and 65%, respectively, for subacromial space ($P < 0.05$) [33-34]. Taking these into considerations, lidocaine administration into the subacromial space may affect the tenocytes when directly injected into the tendon mid-substance; therefore, careful administration of lidocaine is necessary before operation.

Limitations

There were some limitations in this study. First, an acute rotator cuff tear rat model was used, which is not applicable to humans with chronic tendinopathy; hence, caution is required when applying the results from the rat model to human clinical situations. Second, the spectrum of the lidocaine concentrations evaluated was narrow. The stumps of human rotator cuff tears are usually covered by fibrin [35]; therefore, the cells may not be exposed to the same concentrations as used in the present study as long as the lidocaine is not injected intratendinously. Third, the influence of lidocaine combined with steroids that are frequently used in subacromial injections was not examined. Fourth, cultured cells from uninjured tendons were used in the present study, which may have had much greater variability similar to the variability in the clinical presentation. Fifth, the present study was an observational study because the pathway mechanisms were not examined. Sixth, the possibility of a phenotype change could not be completely excluded because the cells behaved differently once they dissociated from their native extracellular matrix. Seventh, we evaluated load and stiffness but not material properties (e.g., stress, modulus), which may add significant uncertainty to the interpretation of findings. Eighth, the sample size in this study was small, which may have caused a type II statistical error. Resolution of these issues would add clarity to our study findings.

Conclusions

This study showed that lidocaine caused cytotoxicity to the tendon fibroblasts *in vitro* and decreased the biomechanical properties in a rat rotator cuff tear model, inducing apoptosis and delaying collagen organization at the tear site. The effect of significant decrease in biomechanical strength with delayed tissue reorganization continued at 4 weeks after surgery; thereafter, biomechanical strength with delayed tissue reorganization returned to baseline levels by 8 weeks after surgery. Although the rat model used in the present study may not be fully representative of clinical settings, we think that subacromial lidocaine injections in patients with rotator cuff tears should be carefully administered before operations.

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Figure Legends

Figure 1

Flow diagram showing how the rats were allocated into the 2 groups and into each experiment

LD: Lidocaine, Cont: Control, MT: Mechanical testing, Histo: Histological analysis, SEM: Scanning electron microscopy

Figure 2

(a): Photograph showing the supraspinatus tendon tear made on the rat shoulder

(b): Scheme of the supraspinatus tendon tear

SSC: Subscapularis tendon, SSP: Supraspinatus tendon, ISP: Infraspinatus tendon, C: Cartilage, M–T: Muscle–tendon junction

(c): Photograph showing the biomechanical testing device

Figure 3

(a): Effects of lidocaine under various concentrations (0.001%–0.1%) on cell proliferation

The histogram shows the mean tenocyte proliferation (%). Error bars represent the standard deviation. The asterisk indicates a significant difference ($P < 0.05$).

(b): Effects of 0.1% lidocaine on cell viability

The histogram shows the mean tenocyte viability (%). Error bars represents the standard deviation. The asterisk indicates a significant difference ($P < 0.05$).

Figure 4

(a): Histogram showing the mean ultimate load to failure (N)

Error bars represent the standard deviation. The asterisk indicates a significant difference ($P < 0.05$).

(b): Histogram showing the mean stiffness (N/mm)

Error bars represent the standard deviation. The asterisk indicates a significant difference ($P < 0.05$).

Figure 5

Picrosirius red staining at the tear site

(a): Photograph showing the lidocaine-exposed tear site 4 weeks after surgery

(b): Photograph showing the phosphate-buffered saline-exposed tear site 4 weeks after surgery (Control).

(c): Comparison of collagen organization between the lidocaine and control groups 2, 4, and 8

weeks after surgery

Error bars represent the standard deviation. The asterisk indicates a significant difference ($P < 0.05$).

Figure 6

TUNEL staining at the tear site 24 h after surgery

(a): Lidocaine group

(b): Control group

Figure 7

Apoptotic cells at the tear site 24 h after surgery evaluated by scanning electron microscopy

(a): Lidocaine group

(b): Control group